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ERRATUM

Through printer's error, paragraph beginning on page 87 in vol. 29, no. 2, is incorrect. The first line should be struck out and the following first line inserted:

"The extremes when all varieties are considered are 8.4 ± 0.50 and"

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ACUTE AND CHRONIC BIOTIN DEFICIENCIES IN THE MONKEY[1] (MACACA MULATTA)

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THREE FIGURES

(Received for publication July 24, 1944)

Biotin deficiency has been produced in animals through the addition of raw egg white or powdered raw egg white to several different diets (Boas, '27; Parsons, '31; Salmon and Goodman, '34; György, '35). Landy ('41) has reviewed the early literature dealing with the experimental and clinical use of egg white in the diet. Recent studies have demonstrated the need for biotin in the diet of certain animals without the use of a biotin-inactivating substance (Hegsted et al., '40, '42; Waisman et al., '42; Ansbacher and Landy, '41; Patrick et al., '41; Cooperman et al., '43; and Waisman and Elvehjem, '43). In the experiments described in this report, biotin deficiency has been produced in the monkey by three methods: (1) limiting the biotin intake over long periods, (2) including egg white in a complete diet, and (3) adding a sulfonamide drug to an adequate diet.

CHRONIC BIOTIN DEFICIENCY

Waisman et al. ('43) demonstrated that monkeys grow normally on a purified diet containing 3% whole liver substance (W.L.S.), liver concentrate powder 1:20 (L.E.), or a solubilized liver residue (fraction L). Fairly good growth was still obtained when the W.L.S. and L.E. were reduced to 1%, but nutritional failure resulted when only 1% fraction L was fed. These results can be correlated with the amount of "folic acid" (Mitchell et al., '41) or "norite eluate factor" (Hutchings et al., '41) present in the supplement (Waisman and Elvehjem, '43).

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[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the National Foundation for Infantile Paralysis, Inc., New York.

We are indebted to The Wilson Laboratories, Chicago, for the liver products; to Dr. A. D. Welch of Sharp and Dohme, Glenolden, Pa., for the sulfa drugs; to Abbott Laboratories, North Chicago, for the halibut liver oil; and to Merck and Co., Rahway, New Jersey, for the crystalline vitamins.

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In the present experiments, animals fed for 6 to 15 months on a basal diet consisting of sucrose 73 parts, purified casein 18, corn oil 3, salts 4, cod liver oil 2, together with the daily addition of thiamine 500 µg., riboflavin 1 mg., pyridoxine 1 mg., calcium pantothenate 3 mg., nicotinic acid 10 mg., choline 100 mg., para-aminobenzoic acid 100 mg., inositol 100 mg., and 25 mg. vitamin C plus 3% fraction L usually showed a thinning of the hair and loss of fur color. Loss of the hair coat may precede the gradual changes in the hair color or vice versa, and may be evenly distributed or limited to definite areas on the shoulders, back or rump. Five monkeys on the same basal diet, but with 3% L.E. showed a less rapid loss of the hair than those fed the fraction L diets. A male animal, monkey no. 1, given the 3% L.E. diet, showed excellent growth over a 33-month period (starting weight 2300 gm., present weight 13,500 gm.), but during the fifteenth and eighteenth months a gradual loss of the fur was observed; after 24 months the remaining fur was uniformly distributed but the color was abnormal; no further hair loss was noted for the next 6 months. This animal was then given 40 µg. biotin methyl ester per day, and after a month there was marked improvement in the fur color on the legs and lower back. Monkeys fed the same purified diet, but with 3% W.L.S. have shown no changes in the fur during a period of 28 months.

The biotin content of the various liver fractions was determined[2] by microbiological assay according to procedures outlined by Shull et al. ('42) and by Lampen et al. ('42). The assays showed that W.L.S. contained 2–3 µg. biotin, L.E. 0.29–1.5 µg. and fraction L 0.23–0.57 µg. per gm. Since the average daily food consumption of the animals is approximately 200 gm. of the 3% liver diets, such monkeys would receive about 6 gm. of the respective liver fractions per day, or 12–18 µg. biotin from the W.L.S. diet, 1.7–9.0 µg. from the L.E. diet, and 1.3–3.4 µg. from the fraction L diet. From the results of 3 years observations on monkeys receiving various liver diets, it can be concluded that the fur loss is directly related to the biotin potency of the various liver products. The present experiments have established that 20 µg. crystalline biotin methyl ester per day is capable of restoring normal hair growth and fur color in monkeys which have been fed for 15 to 24 months a diet containing suboptimal amounts of biotin (figs. 1 and 2). No attempt has been made to determine the exact biotin requirement; but during a portion of this study, 13.8 µg. were fed per day with partial responses in hair growth, and when the biotin was increased to 20 µg. a noticeable improvement resulted.

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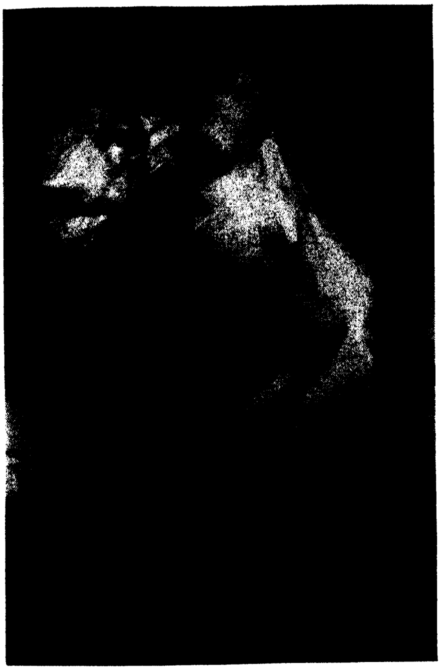
[2] Several biotin assays were kindly made by Dr. J. Gardner, Dr. J. O. Lampen and Mr. D. Miller.

In a previous paper (Waisman and Elvehjem, '43) the experimental findings on two animals which received 1% fraction L, but no added calcium pantothenate, were described in detail. One lost its fur within 150 days; when subsequently fed 3% fraction L, the weight increased, but the fur was still sparse. When the other monkey received "folic acid" concentrate equivalent to 5 gm. fraction L, but no biotin, the fur coat improved temporarily, but after 2 to 3 months large denuded areas reappeared, indicating that the "folic acid" concentrate was of little value in permanently restoring the fur. The "folic acid" concentrate or higher levels of fraction L, were able only to delay the early loss of fur.



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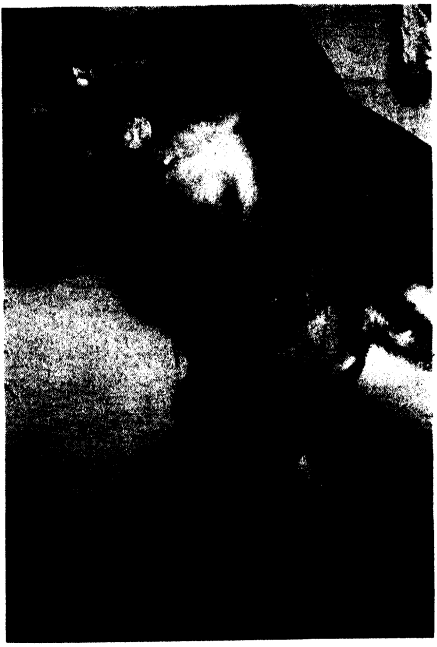

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Fig. 1 Monkey no. 61. Fed low biotin diet (M — 3 + 1% fraction L) (chronic deficiency).
Fig. 2 Monkey no. 61. Three months after feeding 20 μg. biotin methyl ester.

Gradual loss of fur has also been noted in monkeys maintained on a thiamine low diet (Waisman and McCall, '44) for several months. A bisulfite-treated liver extract was included in the diet to provide "folic acid" and other unknown factors. The bisulfite treatment reduced the thiamine content of the L.E. to a barely detectable amount, and had little destructive action on the biotin content as shown by microbiological assay. The fur loss, however, was noted much sooner (8–12 months) than in animals which received the untreated liver extract. This was due in part to the decreased food consumption in those animals maintained on borderline levels of thiamine.

Less marked chronic biotin deficiency was manifested by the appearance of the typical "rusty deposit" (presumably porphyrin-like substances — (McElroy et al., '41; Chick et al., '40) on the abdomen and groin. No skin changes or loss of fur were observed during the course of a year's study in those thiamine deficient animals in which "folic acid" concentrate, and crystalline biotin were substituted for the bisulfite-treated liver.

A definite relationship exists between hair growth and hormonal balance in monkeys. Fur loss was rapid during the attainment of puberty and menstruation in the females fed a biotin-low diet for long periods. When crystalline biotin was fed, female animals which had been undergoing sexual changes and had reached puberty showed a slower regrowth of fur than did immature females or males. In several adult females, the growth of hair seemed to follow the area of extreme redness, and the fur was much more abundant about the hips and legs than on the back and arms. In females with extensive hyperemic menstrual folds along the sides of the legs and around the hips, the hair on this area was never lost completely. Monkey no. 16 had reached puberty shortly after arrival in the laboratory and throughout 2 years of subsistence on various liver extract diets showed marked hyperemia of the eyebrows and continual redness of the perianal region. After a year or more on 3% L.E. the fur became dull and grayish looking, and 6 months later the animal had a sparse fur coat. Twenty micrograms of crystalline biotin were fed daily at this time, and after 2–3 months, a definite return of hair growth and color was evident. Four months later this animal still had redness on the bony prominences around the eyebrows and the hair had regained its color, lustre, and texture. Throughout the long course of biotin therapy, the sexual changes and menstrual folds persisted in intensity as before, but the time necessary for fur regrowth in this animal was undoubtedly related to the increased hormonal activity. Similar observations have been made in six female monkeys.

ACUTE BIOTIN DEFICIENCY

Egg white experiments

The earliest observations on the effect of dried egg white in experimental diets were made about 1927 by several groups of workers and a review of the literature on this subject has been presented by Landy

('41). Three independent and parallel lines of investigation, which dealt with the growth factors required by both rats and bacteria, finally merged so that the relationship among vitamin H, coenzyme R and biotin was clarified (György et al., '40). The review by du Vigneaud ('42) furnishes a concise presentation of the early literature and historical development of the subject.

Early experiments on egg white "toxicity" were in part complicated by diets inadequate in several factors. With this in mind our monkeys on the egg white diet received adequate quantities of all the known vitamins and all the unknown factors which could be supplied by L.E. A diet consisting of sucrose 69 parts, powdered hens' egg albumin 10,

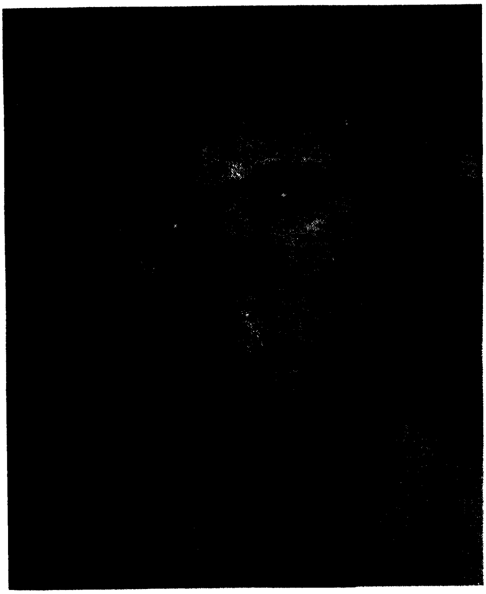

Fig. 3 Monkey no. 83. Acute biotin deficiency (M — 3 + 2% L.E. + 10% egg white).

purified casein 10, L.E. 2, salts 4, corn oil 3, cod liver oil 2, thiamine 500 μg., riboflavin 1 mg., pyridoxine 1 mg., calcium pantothenate 3 mg., nicotinic acid 10 mg., choline 100 mg., para-aminobenzoic acid 100 mg., inositol 100 mg., and ascorbic acid 25 mg. was given to six monkeys nos. 49, 83, 84, 85, 104, and 105. Three of these animals showed signs of acute biotin deficiency within 55 to 100 days; one showed dematitis at 21 days, and two others showed symptoms after the 105th day on the diet. The acute deficiency gave a more severe dermatitis around the face, hands, and feet than was observed in the chronic deficiency (fig. 3). The eyes watered excessively and became red, and incrustations appeared on the eyelids. Some loss of fur color was observed in those monkeys fed the egg white diet for as long as 4 months. Food intake was normal at first but decreased after several weeks. A loss in weight occurred only after the first signs of dermatitis appeared. These signs of acute biotin deficiency are identical with those described by Lease et al. ('37) in two monkeys fed 20% and 40% egg white. The removal of the egg white from the diets given our monkeys and restoration of purified casein to a level of 18% caused a gradual return to normal. Improved appetite was followed by a slow clearing of the dermatitis of the face, hands, and feet, until a new smooth skin appeared in the affected area; the fur color returned to normal and the body weight increased.

The daily administration of 20 μg. of crystalline biotin methyl ester to monkeys receiving egg white caused a rapid return to normal. Eakin et al. ('40) showed that 1 gm. of egg white is capable of inactivating approximately 2.2 μg. of pure biotin; so that the 10% egg white in the diet theoretically makes 22 μg. unavailable to the animal. The addition of 20 μg. of crystalline biotin was evidently sufficient to overcome the harmful action of that quantity of egg white in the diet.

Monkeys which had acute biotin deficiency seemed susceptible to intercurrent infection since various necrotic foci around the face, and at the margin of the teeth and gums persisted throughout the period of egg white feeding. Monkey no. 49 had a small infection of the upper right gum, which gradually became necrotic as the deficiency progressed. In monkey no. 84, a deep crack appeared in the dermatitis of the left leg just below the ankle, which became infected, but improved quickly after daily administration of 20 μg. of biotin. Caldwell and György ('43) and Trager ('43) have recently studied the role of biotin in resistance to infection.

Egg white injury in man has been described by Sydenstricker and coworkers ('42) who found a grayish pallor of the skin, anemia, atrophy of the lingual papillae, and increasing dryness of the skin, which progressed to a maculosquamous dermatitis of the neck, hands, arms, and legs. Decreased urinary biotin excretion was observed after 7 weeks on the egg white diet and neurological signs were also prominent. All these symptoms regressed after administration of 75 to 300 μg. of biotin in the form of a commercial concentrate. Rhoades and Abels ('43) found no signs of biotin deficiency in two patients fed 1000–1200 avidin units (375 gm. frozen egg white and 165 gm. dried egg white) per day for 30 weeks. Oppel ('42) has shown that the biotin content of the urine is influenced by the amount of biotin in the diet, and that

the total biotin output in humans is 3–6 times as great as the intake from the diet.

Sulfa drug experiment

Sulfa drugs have been used in nutritional experiments to inhibit the growth of intestinal bacteria, and thereby prevent bacterial synthesis of certain dietary essentials which are in turn utilized by the host (Black et al., '42; Daft et al., '42; Ransone and Elvehjem, '43). Both sulfaguanidine and sulfasuxidine (succinylsulfathiazole) have been used in attempts to negate the influence of intestinal synthesis, and thus produce one or more dietary deficiencies in a shorter time.

Monkey no. 28 received 1% sulfaguanidine or 1% sulfasuxidine at different times, and each drug retarded growth with equal effectiveness. The loss in body weight was overcome by feeding a "folic acid" concentrate; but after a period of 11 months signs of chronic biotin deficiency were observed, since no biotin was added to the diet at any time; this hair loss was accompanied by the "rusty" secretion on the abdomen and a distinct dryness of the skin. Monkey no. 32 received 1% sulfaguanidine plus 3% L.E. in addition to the basal diet for nearly 7 months, at which time the liver extract was discontinued. The 1% sulfaguanidine was fed for another 2 months with no change in the condition of the animals. One per cent sulfasuxidine was now substituted for the sulfaguanidine and after 3 months the fur color altered and the hair began to fall out. The L.E. feeding presumably deferred the loss of hair. Areas of denudation were accompanied by dryness of the skin and slight reddening of the eyes similar to mild signs of deficiency. It appears that either 1% sulfasuxidine or 1% sulfaguanidine is inadequate to give the severe signs of acute deficiency observed when egg white is fed.

Monkeys nos. 49 and 83 received the basal diet with 1% liver extract and 1% sulfasuxidine plus a daily vitamin supplement without para-aminobenzoic acid (PABA). Monkey no. 83 gained weight consistently, and attained puberty normally during this period, although a definite dryness of the hands and feet was seen at the end of 5 months. The control animal, no. 49, which received 20 µg. of biotin, was normal in appearance and showed improvement of the fur.

Monkeys nos. 84 and 85 received the basal diet plus 1% liver extract and 3% sulfasuxidine without PABA; within 2½ months dryness of the skin appeared on the mouth, nose, arms, and legs, followed 2 weeks later by severe incrustations of the skin. The animals gradually lost appetite, and after 4 months on the diet, deep cracks appeared in the heavy scaly dermatitis of the face. Administration of 20 μg. of crystalline biotin methyl ester per day to monkey no. 85 gave a dramatic cure of all signs of the dermatitis within a 2–3-week period. New-looking smoooth skin appeared as the incrustations fell off, leaving an area through which the skin markings were clearly visible. After 2 months of biotin therapy this monkey grew a new coat of fur. Monkey no. 84 received 100 mg. of PABA per day; but 30 days later showed only slight improvement and after 60 days, the dermatitis was worse and blood appeared at the cracks on the face.

Black et al. ('42) found that PABA counteracted the effect of ½% sulfaguanidine fed in the diet of rats, and Welch et al. ('43) showed that PABA is unable to overcome the effect of sulfasuxidine in rats. PABA is also unable to counteract the effect of sulfasuxidine in the diet of monkeys as seen from the data given in the present report. Although the diet contained only 1% liver extract as a source of "folic acid" it seems unlikely from other data that insufficient "folic acid" would affect the course of the biotin deficiency disease. Experiments are now in progress in which 3% sulfasuxidine is fed together with "folic acid," equivalent to 10% solubilized liver extract.

Complete blood counts[3] were made on the monkeys receiving sulfa drugs in the diet, but no significant changes were noted in the hemoglobin, red blood cell, total white blood cell, and differential white cell determinations during acute biotin deficiency. Axelrod et al. ('43) and Spicer et al. ('42) found granulocytopenia in rats receiving sulfa drugs but their animals were on a synthetic diet without any liver fractions.

The prothrombin time[4] of 12.5% plasma from monkeys fed 1% and 3% sulfasuxidine was not appreciably changed from the normal values. This finding is in contrast to the increased prothrombin time found in rats fed 0.5% sulfaguanidine or sulfasuxidine by Black et al. ('42) and Welch et al. ('43); however, our monkeys were receiving liver fractions.

The addition of 1% sulfasuxidine to the diet, did not seem to aid the monkeys in resisting intercurrent infections which usually accompany the "folic acid" deficiency (Waisman and Elvehjem, '43). Two of the animals fed the drug with the basal diet showed signs of the deficiency, and as the disease progressed the gums became infected and small foci of necrosis were apparent at the tooth line; with the administration of "folic acid" the infection improved.

[3] The authors are indebted to Mrs. Edith S. Jones for help in the blood studies and to Mr. Jack Cooperman for his cooperation in parts of these studies.

[4] We are indebted to Dr. John B. Field and Professor K. P. Link for determining the prothrombin time in our monkeys.

DISCUSSION

The role of intestinal bacterial synthesis in providing biotin to the host is a factor that must be recognized, but since biotin deficiency does occur after long periods it is evident that inadequate amounts of biotin are made available through this means. Intestinal synthesis may also be a factor in the acute biotin deficiency which is produced by feeding 10% egg white. The severe dermatitis is gradually improved after the egg white is removed from the diet, indicating that biotin is slowly synthesized in the tract. The intestinal bacteria were presumably active and unchanged, but the synthesized biotin was immediately made unavailable by the presence of avidin in the egg white. The amount of biotin necessary for normal body function and for the prevention of fur loss is approximately 20 µg. per day. Intestinal bacteria are evidently unable to supply the total daily biotin requirement.

The ability of "folic acid" concentrates to delay or temporarily nullify the antagonistic action of the sulfa drugs has not been adequately explained. "Folic acid" preparations contain PABA which overcomes sulfaguanidine effects, but "folic acid" itself, may be a specific antagonist to sulfasuxidine action. On the other hand "folic acid" may be simply the essential factor for bacterial growth in the presence of sulfasuxidine so that small amounts of biotin may be synthesized and available to the host. Biotin can overcome the host growth inhibition of sulfa drugs in rats (Nielsen and Elvehjem, '42), but it is not known whether biotin is the missing factor per se, or whether biotin is a growth essential for intestinal bacteria enabling synthesis of still another substance required for growth of the host, or fur growth in the case of monkeys. Another point of interest in the problem of vitamin interrelationships is the observation that chronic biotin deficiency appears to be unaffected by a simultaneous pantothenic acid deficiency; the loss of fur is neither hastened nor retarded in the combined deficiency.

Our observations on the relationship of fur growth and hormonal balance in monkeys find support in the observations of Gardner and DeVita ('40), Mulligan ('43), and in Queries and Minor Notes (J. Am. Med. Assn., vol. 121, p. 274, 1943).

The loss of fur in chronic biotin deficient monkeys occurs throughout the year, and the season appears to be without influence on the denudation or regrowth of the fur.

SUMMARY

A chronic biotin deficiency in the monkey produces a thinning of the fur coat and a gradual loss of color in the hair. The time elapsing

before the hair loss in monkeys fed whole liver, liver extract, and solubilized livers can be correlated with the biotin content of the respective liver diets. Twenty µg. crystalline biotin per day was sufficient to cure or to prevent the denudation.

The hair loss is independent of the season, but is affected by hormonal influences, as indicated by slower regrowth of fur in menstruating and adolescent females.

The acute biotin deficiency produced by feeding egg white is strikingly similar to the acute deficiency which results when 3% succinylsulfathiazole is included in a complete diet. Heavy, scaly, dermatitis covers the whole body in the later stages, but is most conspicuous on the face, arms and legs. Twenty micrograms of biotin per day was sufficient to overcome the deleterious effects of the egg white and of the sulfa drugs.

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THE IMPORTANCE OF COMMERCIAL PROCESSING FOR THE PROTEIN VALUE OF FOOD PRODUCTS

I. SOYBEAN, COCONUT AND SUNFLOWER SEED

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In studies of the protein values of food products the emphasis seems to be shifting from the protein values of individual foods to the effect upon these values of the commercial processes used in preparing them for human consumption and in storing them for future use. It is true that individual foods show wide differences in the nutritive value of their proteins. Expressed in terms of biological values as determined upon growing rats at a dietary level of approximately 10%, the proteins of the many foods studied range from 40 to 95. But when combined into diets as complicated as are commonly consumed in this country, the biological values of the proteins of such mixed diets, when reasonably well balanced, may be expected to be much less variable, perhaps falling generally, in the same terms, between 60 and 75. In other words, the combination of foods into diets has a leveling effect on the protein quality, but in such a way that the quality of the mixed proteins will be higher than the mean quality of the individual proteins to the extent that supplementary effects operate.

The most important agency modifying the nutritive value of the proteins of foods during processing is heat. The effect of processes involving heat upon the digestibility and biological value of protein is especially important in human nutrition, but whether the effect of heat will be large or small, or whether it will impair or increase the nutritive value of a given protein source is quite unpredictable on the basis of available information.

The sensitivity of cereal proteins to heat has been known since the experiments of Morgan ('31), but the subject has come into prominence recently in connection with the effect of the extreme heat, applied for only a short time, in the explosion process of preparing certain cereal breakfast foods. Stewart, Hensley and Peters ('43) have shown in feeding experiments on growing rats that the ratio of gain in weight to protein consumed, when the food intakes of the rats are equalized, or nearly so, is depressed by two-thirds by the gun-explosion of an oats-corn-rye mixture, and by three-fourths by the gun-explosion of oats alone. Evidently the preparation of these breakfast cereals yields a product whose protein value in nutrition bears little if any relation to that of the raw cereals from which they were derived. In the public interest, it would seem to be desirable to process cereals for human consumption by methods that are less destructive of nutrient content.

The effect of heat upon the proteins of the leguminous seeds is to improve the nutritive value of the proteins, either by marked improvements in digestibility, as in the navy bean (Waterman and Johns, '21) and the velvet bean (Waterman and Jones, '21), or by a marked improvement in biological value associated with only a slight improvement in digestibility, as in the case of the soybean (Hayward, Steenbock and Bohstedt, '36, and many others), or by means hitherto uninvestigated, as in the case of the lima bean (Finks and Johns, '21) and the lentil (Jones and Murphy, '24).

In cases where heat improves the protein value in nutrition of a given food protein, evidently the intensity of the heat treatment and the time of application will determine the degree of improvement. A too severe heat treatment will undo the favorable effects of a milder treatment. The work of Mitchell, Burroughs and Beadles ('36) demonstrating the refractoriness to commercial roasting of the proteins of the peanut and the relatively low biological values of the protein of both raw and roasted peanuts, 58 and 56, respectively, is not inconsistent with the experiments of Jones and Divine ('44) indicating that expeller process peanut meal contains protein with a growth-promoting value comparable to that of expeller process soybean meal. The roasting of peanuts may represent a much more severe heat treatment than oil extraction by the expeller process, so severe as to nullify the improvement in biological value induced by a milder heat treatement.

It seems of interest to determine quantitatively the effect of heat treatment on the proteins of the soybean, and particularly the effect of the explosion process that is so destructive of the cereal proteins.

The effect of heat processing on soybean protein

The digestibility and the biological value of the protein in raw soybeans of the Illini variety and of the same sample of soybeans heated in the autoclave for 1 hour at 15 pounds steam pressure were determined by the nitrogen metabolism method used in this laboratory (Mitchell and Carman, '26), with some later modifications. These modifications include the use of a feces marker (Fe_2O_3 or Cr_2O_3), a reversal system of feeding whereby each rat receives each of the two protein sources to be compared, five rats in one order and the other five in the reverse order with the standardizing period in between, and a system of paired feeding, treating the ten rats as five pairs within which the food intake is equalized.

The experimental rations in this test contained 12.5 to 13.1% of total protein (N × 6.25), or 10 to 11% of digestible protein. In other respects they were adequate for maximum growth. The water-soluble vitamins were added as Harris' yeast concentrate.

The true digestibility and the biological values, computed in the usual way, are summarized in table 1.

TABLE 1

A comparison of the utilization of protein in raw and autoclaved soybeans.

RAT NUMBER	RAW SOYBEAN MEAL		AUTOCLAVED SOYBEAN MEAL	
	True digestibility of protein	Biological value	True digestibility of protein	Biological value
	%	%	%	%
75 m	78	52	83	72
77 m	72	48	83	63
79 m	78	47	86	70
81 m	76	45	83	62
83 f	76	49	79	76
76 m	81	52	85	67
78 m	74	44	80	66
80 m	81	52	88	64
82 m	83	51	82	64
84 f	81	51	82	66
Average	78.0	49.1	83.1	67.0

There is evidently a statistically significant difference between autoclaved and raw soybean meal in both the digestibility of the nitrogen and in its biological value. Autoclaving has increased the digestibility of the soybean nitrogen by some 5 percentage units and the biological value by 18 percentage units. It may be noted that the average results in this experiment on autoclaved soybeans check remarkably well with the results of an experiment carried out 6 years later (Mitchell and Beadles, '44) on Illini soybeans autoclaved for 1.5 hours at 17 pounds steam pressure. In the latter test the average true digestibility of nitrogen, corrected, as are the values in table 1, for the metabolic nitrogen in the feces, is 84.4%, and the average biological value, 67.5.

In paired-feeding experiments on young growing rats, it was shown by Mitchell and Smuts ('32), and later by others, that cystine is the amino acid limiting the utilization of raw soybean proteins in metabolism. In later paired-feeding experiments, the details of which need not be given here, it was shown that cystine also limits the utilization in metabolism of the autoclaved soybeans used in the tests summarized in table 1. With eight pairs of rats (45 to 50 gm. initial weight) on diets containing 10% of protein, the rat in each pair receiving a cystine supplement gained at the more rapid rate; the mean difference in gain in a 2-week feeding period was 6.4 gm., the standard deviation of differences was 2.55 gm., and the probability of a fortuitous outcome was so small (.0002) that it can safely be neglected (Student, '08).

In another series of tests with eight pairs of rats, 10% of raw soybean protein plus 0.25% of cystine was compared with 10% of autoclaved soybean protein plus 0.25% of cystine. In all pairs but one, the rat receiving autoclaved soybeans gained the faster in a 5-week feeding test. The mean difference in gain induced by the two diets was 8.1 gm., the standard deviation of differences was 5.35 gm., and the probability that a random combination of uncontrolled experimental factors could have brought about such a result is only 0.0025, and can safely be neglected. Repetition of the tests with older rats and with a supplement of 0.4% of cystine instead of 0.25% failed to reveal a significant difference in growth-promoting power of autoclaved over raw soybeans in a 2-week feeding test. The latter test was not entirely satisfactory because of the heavier initial weights of the rats, 74 to 109 gm., and the less perfect initial pairing.[1]

The conclusion seems justified, however, that the effect of the heating treatment in these particular tests was to render soybean protein more digestible and more completely utilizable in metabolism, the latter effect being due solely to an improved availability of the cystine contained in the soybean protein.

To determine the effect of the explosion process upon the nutritive value of the protein of the soybean, we obtained three samples of soybean flour all taken from the same basic lot of soybeans.[2] These samples were a raw soybean flour, an exploded soybean flour and a partially exploded soybean flour.

The raw soybean flour was not submitted to any heat treatment other than that involved in grinding.

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[1] The paired-feeding tests referred to in this and in the preceding paragraph were carried out by Dr. A. J. Johanson, then a graduate student in animal nutrition.

[2] These samples were obtained through the Musher Corporation of New York City, which contributed funds for the carrying out of these tests.

In the preparation of the exploded soybean flour, the beans were placed in a rotating closed chamber, or "gun", at a temperature of approximately 550°F. for a period of 90 seconds. Steam was then injected into the gun for an additional period of 60 seconds until the steam pressure reached 185 pounds per square inch. At the end of this time, the gun was suddenly opened so as to produce an instantaneous drop in temperature and pressure to atmospheric conditions.

The partially exploded soybean flour was prepared exactly as described for the fully exploded flour except that the steam was injected over a period of 45, instead of 60 seconds, and the pressure reached was 165 pounds per square inch, instead of 185 pounds.

The chemical analysis of the three flours, summarized in table 2, revealed a remarkable similarity in composition.

TABLE 2

Chemical composition of raw and exploded soybean flours.

SAMPLE	DRY MATTER	ETHER EXTRACT	CRUDE PROTEIN	TOTAL NITROGEN	CRUDE FIBER	GROSS ENERGY
	%	%	%	%	%	*cal./gm.*[1]
Soybean flour — Exploded	94.81	21.90	39.62	6.34	2.51	5408
Soybean flour — Raw	93.20	20.70	39.62	6.34	2.49	5339
Soybean flour — Partly exploded	94.56	22.96	39.69	6.35	1.65	5536

[1] Small calorie per gram.

The true digestibility and the biological value of the nitrogen in the three flours was compared in each case with those of a defatted and dehydrated beef, prepared at low temperatures. Ten young growing rats, weighing initially 50 to 55 gm. each, were used in each comparison, which consisted of three collection periods. In the first period, five rats received the soy flour ration and their pair mates received the beef ration. In the second period, all rats received the 4% egg protein diet as a standardizing ration, and in the third period the beef and soy flour rations were again compared but in reverse order, so that in the first and third periods each rat was tested with both rations. Throughout these periods pair mates consumed the same amount of food. Each collection period was preceded by a period of at least 5 days on constant food.

The composition of the experimental diets is given in table 3. They were equalized in fiber and energy content, and except for the standardizing egg-protein diet, in nitrogen content. The true digestibility and the biological values of the nitrogen of the three flours are collected in table 4.

The almost identical average values for the digestibility and biological value of the protein of beef in the three experiments testifies to the accuracy of the method, discussed in a previous publication (Mitchell, Burroughs and Beadles, '36), and the essential accuracy of the conception of protein metabolism upon which the method is based (Mitchell, '42; '44). The shortness of the metabolism period involved in this method has been recently criticized, because of the possibility "that a suboptimal concentration of a particular amino acid in the protein, which would ultimately prove to be a handicap to the animal, might be without consequence for the nitrogen balance during a short test period."[3] This hypothetical situation seems inherently improbable. The amount of amino acids stored in the tissues is extremely small and its replenishment from the tissue proteins for the purpose of supplementing inadequate dietary protein would involve losses of nitrogen that would immediately be reflected in the nitrogen balance of the animal. As a matter of fact, the rat (Burroughs, Burroughs and Mitchell, '40) and other

TABLE 3

The experimental diets in the soybean flour tests.

CONSTITUENTS	BEEF RATION	EGG STANDARDIZING RATION	SOY FLOUR, EXPLODED, RATION	SOY FLOUR, PARTLY EXPLODED, RATION	SOY FLOUR, RAW, RATION
Dried defatted beef round	11.83	..	..	..	..
Dried defatted whole egg	..	5.75	..	..	..
Soy flour, exploded	..	..	25.24	..	..
Soy flour, partly exploded	..	..	..	25.20	..
Soy flour, raw	..	..	..		25.24
Salt mixture no. 446	4	4	4	4	4
NaCl	1	1	1	1	1
$BaSO_4$ (or wood flock)	2	2	1.37	1.58	1.37
Vitaminized starch[1]	5	5	5	5	5
Starch	54.83	60.58	46.92	47.01	46.61
Fortified cod liver oil	1.5	1.5	1.5	1.5	1.5
Wheat germ oil	0.5	0.5	0.5	0.5	0.5
Soy oil	5	5	..	..	..
Lard	4.34	4.67	4.47	4.21	4.78
Sucrose	10	10	10	10	10
Total	100.00	100.00	100.00	100.00	100.00
Crude protein content, %	9.82	4.31	10.06	10.25	10.31

[1] The vitaminized starch contained per gm., 20 µg. of calcium pantothenate, 60 µg. of pyrdoxine HCl, 80 µg. of riboflavin, 50 µg. of thiamine HCl and 30 mg. of choline HCl.

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[3] Nutrition Reviews, vol. 2, no. 7, p. 213.

TABLE 4

True digestibilities and biological values of the protein of the three soy flours, each compared with the protein of beef round.

EXPERIMENT NUMBER 335

RAT NO.	Beef round		Soy flour, exploded	
	True digestibility	Biological value	True digestibility	Biological value
11	100	76	93	65
13	100	75	92	72
15	100	69	92	66
17	100	77	93	74
19	100	76	91	77
12	99	81	95	74
14	98	82	93	71
16	98	80	96	68
18	99	85	96	73
20	98	81	93	72
Averages	99.2	78.2	93.4	71.2

EXPERIMENT NUMBER 336

RAT NO.	Beef round		Soy flour, partly exploded	
	True digestibility	Biological value	True digestibility	Biological value
21	100	74	93	77
23	100	67	94	75
25	100	70	95	80
27	100	76	96	75
29	100	84	94	78
22	100	82	95	66
24	100	81	98	79
26	99	82	96	72
28	100	80	97	72
30	100	85	98	78
Averages	100	78.1	95.6	75.2

EXPERIMENT NUMBER 339

RAT NO.	Beef round		Soy flour, raw	
	True digestibility	Biological value	True digestibility	Biological value
41	100	86	84	58
43	100	82	86	71
45	100	77	87	65
47	100	77	86	60
49	100	77	85	60
42	98	77	84	55
44	100	75	87	57
46	99	78	85	56
48	98	72	83	56
50	99	80	81	56
Averages	99.4	78.1	84.8	59.4

animals respond promptly in their balance of nitrogen to the withdrawal from the diet of an essential amino acid.

The average digestibility and biological value of the nitrogen of the raw soy flour, 84.8 and 59.4, respectively, were significantly lower than those of the fully exploded flour, 93.4 and 71.2, respectively. This relationship proves that the more severe explosion treatment has definitely improved the nutritive value of the soybean protein to even a greater extent than the autoclaving practiced in the preceding soybean experiments.

The protein of the partially exploded soy flour is definitely more digestible than that of the fully exploded flour, 95.6 as compared with 93.4, with a probability of only 0.021 (Fisher, '28) that chance factors alone could have brought about this spread. The biological value of the protein in the partly exploded flour averaged higher than that of the fully exploded flour, 75.2 as compared with 71.2, but here the difference is not sufficiently distinct in comparison with the intra-group variation (P = 0.055) to constitute a demonstration. However, the results are highly suggestive that here also the less severe heat treatment has been definitely advantageous in producing a more favorable effect on the nutritive value of soybean protein.

Mild processing methods preserve the protein value of the coconut and sunflower seed

The commercial extraction of oil from oil-bearing seeds is ordinarily carried out by procedures that give the highest yields of oil with minimal refining losses. Little attention is paid to the nutritive value of the oil meal, which is commonly used for stock feed. Whether the oil is extracted by pressure or by solvent, high temperatures are commonly employed, either in the pretreatment of the seeds, the extraction process itself, or in the removal of the solvent. Inevitably the protein value of the residual oil meal is impaired (except for the soybean) unless special precautions are taken. Olcott and Fontaine ('44) have demonstrated the injurious effects of heat on the proteins of the cottonseed, such as probably result from commercial oil extraction. The variable results that have been reported for the biological value of the proteins of cottonseed meal, ranging from 62 (Nevens, '21), through 78 (Braman, '31–'32) to 81 (Smuts and Malan, '38), may be explainable by variations in the heat treatment employed in commercial oil extraction.

The same situation appears to exist for the coconut and the effect of oil extraction on the protein value of coconut meal. In 1919, Johns, Finks and Paul reported on the growth-promoting value for rats of the proteins of coconut press cake, and found that diets containing 13.1% of coconut protein (N × 5.7) were capable in some cases of supporting a growth rate of 1.5 to 2.0 gm. daily, which at that time was considered normal. Somewhat later, Maynard and Fronda ('21) gave to the proteins of coconut oil meal a value somewhat higher than that shown by the proteins of corn, measuring these values as gains per gram of protein consumed in ad libitum feeding experiments. However, the values reported on both foods were few and variable. Mitchell and Villegas ('23), using the nitrogen balance method, found an average biological value of 58 for the proteins of coconut meal, quite comparable with the values commonly found for corn by this method (Mitchell, '24). Using the same method, Smuts and Malan ('38) reported an average biological value of 69 for the proteins of "coprameal".

The opportunity of testing the protein value of a coconut meal that was defatted by a solvent extraction method involving low temperatures throughout (less than 75°C.) was afforded us through the courtesy of Mr. Ezra Levin of Monticello, Illinois.[4] The meal was finely ground, very light in color and possessed only a slight coconut odor. At the same time we secured also a sample of sunflower seed meal prepared by the same process and by the same company. Both meals appeared to be edible, capable of being incorporated in flour mixtures for baking. Their chemical composition is given in table 5.

TABLE 5

Chemical composition of the sunflower seed meal and the coconut meal.

	DRY MATTER	ETHER EXTRACT	CRUDE PROTEIN	ASH	CRUDE FIBER	N-FREE EXTRACT	GROSS ENERGY
	%	%	%	%	%	%	*cal./gm.*
Sunflower seed meal	95.41	4.48	52.83	6.46	4.05	27.59	4.57
Coconut meal	95.21	10.25	19.69	5.19	9.39	50.69	4.31

Both meals were incorporated into rations containing approximately 10% of protein (N × 6.25) and compared by the usual procedure, as explained above, with the proteins of beef, using young albino rats as subjects. The true digestibilities and the biological values secured are summarized in table 6.

Noteworthy, is the high average biological value of 71 for the coconut meal protein, as compared with the value of 58 previously obtained from this laboratory on a commercial meal presumably subjected to a much higher heat treatment. The average biological value of 64.5 for the proteins of sunflower seed meal places this protein source in the same class as the better cereals, oats, wheat and barley. The digestibility of sunflower seed proteins is definitely higher, 94.3, than that of coconut proteins, 86.1, but even the latter is not unusually low, as compared with cereal proteins, for example.

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[4] Of the VioBin Corporation, which provided funds to cover in part the expenses of this study.

TABLE 6

The digestibility and biological value of the protein of sunflower seed meal and coconut meal, compared with the protein of beef round.

EXPERIMENT NUMBER 342

RAT NO.	Beef round		Sunflower seed meal	
	True digestibility	Biological value	True digestibility	Biological value
51	99	83	94	66
53	100	84	95	63
55	97	66	93	62
57	98	79	96	64
59	100	81	94	65
52	97	77	91	61
54	98	77	94	64
56	97	73	92	63
58	100	79	99	69
60	99	81	95	68
Averages	98.5	78.0	94.3	64.5

EXPERIMENT NUMBER 343

RAT NO.	Beef round		Coconut meal	
	True digestibility	Biological value	True digestibility	Biological value
61	99	72	87	68
63	99	74	89	70
65	99	71	88	70
67	99	69	87	66
69	94	69	88	75
62	98	60	80	70
64	97	86	86	77
66	96	77	87	71
68	98	77	87	65
70	98	83	82	75
Averages	97.7	75.8	86.1	70.7

The method of oil extraction at comparatively low temperatures, such as was used with these products, and with corn germ (Mitchell and Beadles, '44) and also wheat germ, has so many apparent advantages in preserving the nutrient content of both oil and meal that it, or some similar method, may well initiate a revolution in the extraction of edible oils from oil bearing seeds. This will be especially true when the oil extraction business, and the consuming public, realize the high nutritive value of many oil-bearing seeds, entirely aside from their oil content. The drastic methods of oil extraction now in general use produce meals often inferior and always variable in protein value. The mild, gentle method, such as was used in the preparation of the meals here studied, produces uniform meals, stable in character, probably with the original nutrients virtually unimpaired in amount and quality,[5] and to all appearances of excellent culinary properties. They are worthy of further study to determine the extent to which they can be introduced into the human diet as sources of protein, thiamine and other nutrients.

The net protein values of the foods studied

The value of a food as a source of dietary protein is not, of course, measured by the biological value. It evidently depends upon three factors, the protein content, the digestibility of the protein and the biological value of the digested protein. Taking all of these factors into consideration, leads to what the senior author has called the "net protein" value (Mitchell and Carman, '24) which possesses obvious advantages in the comparative ranking of protein foods. The net protein values of the food products discussed in this report have been computed and summarized in table 7.

TABLE 7

The net protein values of the foods studied in these experiments, on the moisture-free basis.

FOOD PRODUCT	PROTEIN CONTENT	DIGESTIBILITY OF PROTEIN	CONTENT OF DIGESTIBLE PROTEIN	BIOLOGICAL VALUE OF PROTEIN	NET PROTEIN VALUE
	%	%	%	%	%
Raw soy flour	42.5	84.8	36.0	59.4	21.4
Partly exploded soy flour	42.0	95.6	40.2	75.2	30.2
Fully exploded soy flour	41.8	93.4	39.0	71.2	27.8
Coconut meal	20.7	86.1	17.8	70.7	12.6
Sunflower seed meal	55.4	94.3	52.2	64.5	33.7

In terms of net protein content, the products arrange themselves in the following order: sunflower seed meal, partly exploded soy flour, fully exploded soy flour, raw soy flour and coconut meal. In terms of vitamin content, the products subjected to the least severe heat treatment would probably outrank products severely heated in commercial processing.

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[5] For example, the thiamine content of a corn germ meal prepared by the Levin process was found to be 25.6 μg. per gram.

SUMMARY AND CONCLUSIONS

Using the nitrogen balance method of assessing the protein values of foods with growing rats, it has been shown that the digestibility and biological value of the proteins of the soybean can be increased by heat processing, and that the explosion process, if not carried to extremes, can raise the digestibility of the protein by 11 percentage units and the biological value by 16 percentage units.

In the autoclaving of soybeans, the improvement in the nutritive value of the protein seems to be entirely referable to an improvement in the availability of the contained cystine.

In the commercial oil extraction of other oil-bearing seeds than the legumes, the drastic heat treatments commonly employed may be expected to exert a destructive action upon the heat labile nutrients, including protein. Applied to the coconut, a solvent extraction procedure carried out at temperatures that never exceed 75°C., yields a product whose protein is 86% digestible and possesses a biological value of 71, considerably higher than that of a product tested earlier that had been prepared by the usual drastic methods.

The protein of sunflower seed meal, prepared by the same process, was found to be 94.3% digestible and to possess a biological value of 64.5. Due to its high initial content of protein, 55.4% on the dry basis, the "net protein" content of this food was higher than that of any of the other foods tested, i.e., 33.7%.

The significance of a mild, gentle process of extracting oil from non-leguminous oil-bearing seeds in the preparation of protein foods for human consumption seems worthy of further study.

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ENRICHED, MORRIS TYPE AND WHOLE WHEAT FLOUR AS SOURCES OF THE B-COMPLEX VITAMINS[1]

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THREE FIGURES

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About one-third of the total calories of American and British diets is obtained from bread and cereals. For European countries other than Britain, this figure is increased to 50% or more. As flour occupies such an important place in the diet, it is desirable that it be of high nutritive value.

It is well known that the whole wheat grain contains more nutrients than patent flour. Several methods have been used to improve the nutritive value of patent flour. Addition of minerals and synthetic vitamins to patent flour, or modifying the milling process so as to retain more of the nutrients of the whole grain, are two means of increasing the mineral and vitamin content of flour. In addition bread may also be improved nutritionally by the use of high vitamin yeast and other supplements. Enrichment of flour is doubly important since it is used in many forms besides bread.

It was shown by Fairbanks ('39) that addition of milk solids to bread increased the nutritive value. Mitchell, Hamilton and Shields ('43) found that white bread enriched with thiamine, niacin, iron and riboflavin did not promote as good growth in rats as when non-fat milk solids were added. Chick ('40) reported that white flour of 73% extraction supplemented with casein, fat, salt mixture, fat-soluble vitamins and pure thiamine did not produce as good growth in rats as whole wheatmeal. Wright ('41) obtained the same rate of growth in animals with 85% wheatmeal and with white flour of 75% extraction fortified with thiamine.

Experiments with young college women by Free ('40) showed an improved state of nutrition when white bread made with high vitamin yeast was ingested. Sealock, Basinski and Murlin ('41) found that the higher "indigestible residues" of whole wheat products did not interfere with the digestion and absorption of carbohydrate and fat. That the consumption of extra B-complex vitamins improves the biological value of proteins was shown by Murlin, Marshall and Kochakian ('41) when they tested the digestibility and biological value of whole wheat and white bread. Sealock and Livermore ('42) reported that peeled wheat bread was a good source of the B-complex vitamins and Alcock and Larmour ('42) were of the opinion that long extraction milling may solve the problem of more nutrients in flour.

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[1] Contribution no. 123, Department of Home Economics.
Contribution no. 110, Department of Milling Industry.

Light and Frey ('43) obtained better weight gains when rats were fed white bread supplemented with lysine, valine, salt mixture, vitamins A, D, E, and riboflavin, than when fed either ordinary white bread or bread enriched at the old levels (previous to October, '43) and without the addition of riboflavin. Williams, Mason and Wilder ('43) made studies with human beings on diets low in thiamine and riboflavin. A supplement of enriched flour plus 6% milk solids gave the same results as whole grain flour. Lepkovsky ('44) in reviewing the bread problem states: "Unlike the English, they (the Americans) have not discussed with any degree of thoroughness the relative merits of the whole wheat bread, white bread and 'enriched' white bread."

For the purpose of gaining further information on this problem growth studies on albino rats were conducted in order to compare the relative values of whole wheat, with a Morris type of flour and enriched flour, as sources of the B-complex vitamins, when these are included as 30, 40 and 50% of the diet.

EXPERIMENTAL PROCEDURE

Young albino rats weighing between 40 to 50 gm. were used for the tests. Each animal was placed in an individual cage on a raised wire screen to prevent consumption of feces. Food and water were given ad libitum. The B-complex free basal diet consisted of vitamin free casein, 20%; cornstarch, 60%; corn oil, 12%; salt mixture, 5%; and cod liver oil, 3%. This was a modified Chase and Sherman ('31) diet. The casein supplied the amino acids that are lacking in wheat or flour, and the salt mixture the additional minerals needed. The cod liver oil furnished vitamins A and D. The rat does not require the addition of vitamins C and K; therefore the B-complex vitamins were the limiting factors.

All animals were fed the B-complex free diet for 12 days before the beginning of the test period in order partially to deplete their body stores of these vitamins. At the end of the depletion period they were divided into groups of five or eight animals and placed on the test diets. The supplementing materials were whole wheat, Morris type of flour, patent flour enriched at the old levels with thiamine, niacin and iron, which will be designated as "old enriched flour" and patent flour enriched at the new or present levels with thiamine, niacin, riboflavin and iron, designated as "new enriched flour."

There were four series of tests. In the first series patent flour, whole wheat, old enriched, and Morris type flour were used as supplements. All these materials were obtained from one mill and one mill mix. The plain and enriched patent flour was of 70% extraction. The whole wheat was finely ground in a burr mill. In order to test wheat and flour from different sources, another mill supplied the flour and wheat for the second and third series of tests, while still another supplied the materials for the fourth series.

The average American diet contains approximately 30% cereals. In some cases this may be increased to 40 or 50%; therefore the supplements were incorporated into the diets at 30, 40 and 50% levels. They replaced an equivalent amount of cornstarch in the basic diet. Weights of the animals were recorded every 6 days. The average weights of the animals on each test material are shown by growth curves.

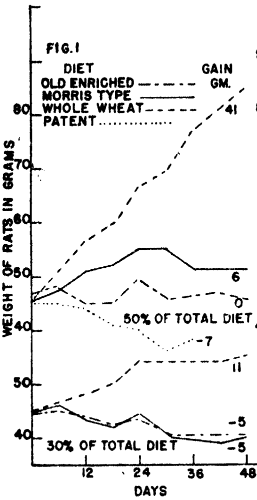
RESULTS

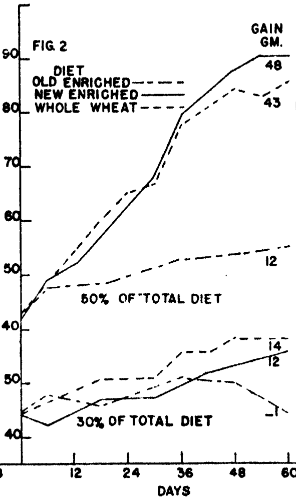
The growth curves show the average weights of the five animals with each supplement at a 30% level (fig. 1). The supplements were whole wheat, Morris type flour and old enriched flour. One group of animals was left on the B-complex free diet. These served as negative controls and showed that the basal diet would not support growth as these animals lost weight and died before the twenty-fourth day.

The animals on the Morris type flour and the old enriched flour showed parallel weight changes and lost an average of 5 gm. during the 48-day test. Those receiving whole wheat gained an average of 11 gm. showing that when fed at a level of 30% of the diet, whole wheat used as a source of the B-complex vitamins, promoted better growth than either the Morris type flour or old enriched flour. They also were more alert and their fur had a better texture.

The animals on whole wheat at the 50% level (fig. 1) gained an average of 41 gm. during the 48-day test period. They had the appearance of healthy rats. When ordinary patent flour was included at this level, the animals lost weight slowly and died by the thirty-sixth day. Those on the old enriched flour weighed on the average the same at the end of the experiment as at the beginning, while those on the Morris type flour made an average gain of 6 gm. and had a better appearance than those on the old enriched flour.

The different materials were analyzed for thiamine content. The wheat from which the Morris type flour was milled contained 5.3 µg. per gram, the Morris type flour contained 3.3 µg. per gram, while the old enriched flour assayed 3.97 µg. per gram. Although the old enriched flour contained more thiamine than the Morris type flour, the animals made slightly better gains on the latter, when both were fed at the 50% level in the diet, showing that other factors besides thiamine are involved.





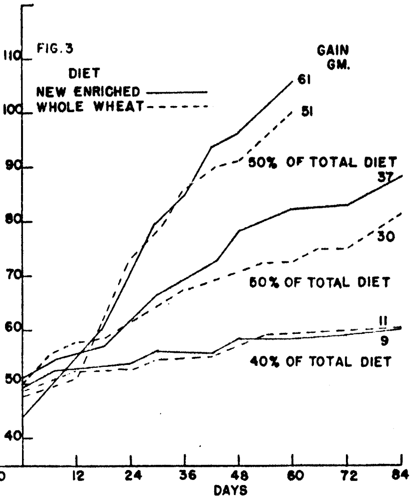


Fig. 1 Growth curves of rats in the first series of tests using flour and wheat as sources of the B-complex vitamins.

Fig. 2 Growth curves of rats in the second series of tests.

Fig. 3 Curves showing the average rate of growth of the rats. The four lower curves show the third test and the two upper curves the fourth test.

A second series of experiments was conducted in the same manner, except that new enriched flour was included. At the 30% level (fig. 2) the animals on the old enriched flour gained on the average 5 gm. during the first 48 days of the test but lost this gain before the sixtieth day. Throughout the test, those on the whole wheat maintained a slightly higher weight gain than those on the new enriched flour. At the end of the 60 days those on the new enriched flour had gained 12 gm. and those on whole wheat 14 gm.

At the 50% levels (fig. 2) the animals on the old enriched flour gained an average of 12 gm. during the entire period while those on whole wheat and new enriched flour made almost parallel gains until the thirty-sixth day, after which those on the new enriched flour made better gains. At the end of 60 days those on whole wheat had made an average gain of 43 gm. and those on new enriched flour had gained 48 gm.

It was decided to run a third series using the same flour and whole wheat as in the second series but with the supplements at the 40 and 50% levels and including a group of animals on the stock diet. This was an 84-day test and eight animals were used in each group (fig. 3). The average weight gains, when the wheat and new enriched flour were fed at the 40% levels, were practically parallel throughout the test. At the 50% level, the curves were parallel for the first 36 days, but by the eighty-fourth day the animals on the new enriched flour had gained an average of 7 gm. more than those on whole wheat. None of these animals grew as well as those on the stock diet. The total average gain of the stock animals during the 84 days was 202 gm.

At the end of the experiment some of the animals on each supplement were autopsied. No excess fat was found in the livers. As compared to the animals on the stock diet, the animals on the test diets had normal organs except for size.

The thiamine content of the whole wheat was 3.58 μg. per gram, for old enriched flour 3.83 μg. per gram, and 3.96 μg. per gram for new enriched flour. The riboflavin values were 1.14 μg. per gram for whole wheat as compared to 0.41 μg. per gram for old enriched flour and 2.56 μg. per gram for new enriched flour. The greatest difference is shown in the riboflavin values. As the new enriched flour contained more riboflavin than either the whole wheat or old enriched flour the use of riboflavin along with thiamine may account for the increased growth of the animals on the new enriched flour. Higgins, Williams and Mason ('43) fed rats a diet low in thiamine, analogous to that consumed by some humans and reported that increasing the thiamine in the bread did not promote better growth but the addition of both thiamine and riboflavin did induce a growth rate equal to that of whole wheat bread.

Even though the growth rate of the rats may have been improved by the addition of riboflavin as well as thiamine and niacin in the new enriched flour, the maximum growth rate thus attained did not equal that of the animals fed the stock diet. The stock diet consisted of a commercial dog food[2] and contained an adequate amount of all the B-complex vitamins. Neither whole wheat or enriched flour contained enough of all of the B-complex vitamins to produce optimal growth, but the addition of thiamine, niacin and riboflavin definitely improved the nutritive value of patent flour sufficiently to make it equal to or better than whole wheat in promoting growth, depending on the level fed.

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[2] Purina Dog Chow.

Wheat and flour obtained from a different mill were used in another series of tests in order to eliminate any errors due to milling processes or mixing of the enriching materials or other factors. Again the flour was milled from the same lot of wheat as was used in the test. The new enriched flour and the wheat were fed at the 50% level in the diet. Five animals were used on each test. The growth curves were parallel for 36 days of the test (fig. 3) at which time the rats on the new enriched flour began to gain at a faster rate than those on whole wheat. At the end of the 60-day period the animals on the new enriched flour had made an average gain of 61 gm. while those on whole wheat had achieved an average gain of 51 gm.

The wheat assayed 4.10 µg. per gram for thiamine, 1.08 µg. per gram for riboflavin and the new enriched flour 4.69 µg. and 3.13 µg. per gram respectively. In this case the enriched flour was higher in both thiamine and riboflavin content than the whole wheat which may be the reason for the difference of 10 gm. in the weight gains of the two groups as compared to a difference of 7 gm. in the first test at the 50% level and 5 gm. in the second test. These differences in the average growth rates between the animals fed whole wheat and new enriched flour seem to be rather definite evidence that flour, enriched at the new levels and with riboflavin, is a better source of the B-complex vitamins than whole wheat when these are fed as 50% of the total diet.

SUMMARY AND CONCLUSIONS

Four series of experiments were conducted on albino rats to compare the relative growth promoting values of whole wheat, Morris type flour, patent flour enriched at the old levels, and patent flour enriched at the new levels, as a source of the B-complex vitamins. The first experiment included whole wheat, Morris type flour and old enriched flour, the second, compared whole wheat, old enriched flour and new enriched flour at 30 and 50% levels in the diet, while the third made comparisons at the 40% level. A fourth test was run on wheat and flour from another mill at the 50% level.

Under the conditions of these experiments, the results indicate that whole wheat is a better source of the B-complex vitamins than either Morris type flour or patent flour, enriched at the old levels, when these materials make up 30 or 50% of the diet. At a 30% level whole wheat is slightly better than patent flour, which has been enriched at the new levels. Whole wheat and new enriched flour promote the same amount of growth when fed at a 40% level, while at a 50% level the new enriched flour is better as a source of the B-complex vitamins than whole wheat.

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THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON THIAMINE REQUIREMENT OF THE RAT

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ONE FIGURE

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The relation in animals of thiamine requirement to carbohydrate metabolism has been extensively studied, and has been discussed in detail by Ochoa ('42). The effect of increased metabolic rate on the need for thiamine, also well known, is pointed out by Cowgill ('39) and Williams and Spies ('38). Environmental temperature, however, in relation to thiamine utilization has received relatively little attention. It may be expected that any environmental factor that influences energy utilization or output will in turn affect the requirement for thiamine. In our work on biological methods for determining thiamine a profound effect of environmental temperature on thiamine requirement has been demonstrated both in rat-curative and rat-growth experiments. It has been found that careful temperature control is essential in obtaining a high degree of accuracy, particularly in the rat-curative type of assay. In these studies the cure of polyneuritis in rats and the growth of rats receiving varying levels of thiamine were used as criteria of thiamine requirement. The animals were kept in air-conditioned rooms with adequate temperature and humidity control.

Studies involving polyneuritis

The rat-curative studies were conducted in accordance with the U.S.P. method of assay for thiamine[1] because there is convincing evidence that the duration of the cure of polyneuritis with the diet prescribed is not influenced by dietary factors other than thiamine. Litters of white rats at the age of 13 days were transferred with their mothers to the U.S.P. thiamine-deficient diet, and were later weaned at a body weight of 40 to 50 gm. The thiamine-deficient diet was fed ad libitum and the animals were kept in cages with wire screen bottoms throughout the experiment. Each litter of rats was kept in one cage until the twenty-fifth day of the depletion period and the animals were then segregated into individual cages. The animals were weighed weekly and after the twenty-fifth day of the depletion period were observed twice daily for symptoms of polyneuritis. At the first and at each successive occurrence of polyneuritis the animals were given a single dose of the U.S.P. Thiamine Hydrochloride Reference Standard by stomach tube. The animals were maintained during the curative periods at a temperature of 78°, 85°, or 90°F., and were transferred from one temperature to another at the time polyneuritis occurred and a dose of thiamine was administered. Size of doses, environmental temperatures, and the resulting curative responses measured in days are given in tables 1 and 2.

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[1] The Second Supplement to the Pharmacopoeia of the United States of America, Eleventh Decennial Revision, p. 129.

TABLE 1

Duration of curative response to 6-µg. doses of thiamine at different environmental temperatures.

RAT NO.	FIRST PERIOD 78° F.	SECOND PERIOD 85° F.	RAT NO.	FIRST PERIOD 78° F.	SECOND PERIOD 85° F.
	days	*days*		*days*	*days*
3534	6	10	3548	10	10
3536	5	11	3524	11	15
3523	9	9	3532	8	10
3501	12	15	Average	8.7	12.4
3503	12	16	$t = \frac{\text{difference between averages}}{\text{standard error of difference}} = 3.096$		
3542	5	15			
3527	9	13	therefore $P < 0.01$		

In a preliminary study involving only four animals kept alternately at 78° and 90° F. over four curative periods it was found that a 6-µg. dose of thiamine produced curative responses of 6 to 9 days duration at 78° F. and 11 to 15 days at 90° F. The duration of the cure did not seem to be influenced by the temperature at which the animal had been kept prior to the administration of the curative dose.

The data presented in table 1 are for ten animals maintained during the first curative period at 78° F. and during the second at 85° F. Although two of the animals had curative periods of equal length at the two temperatures, the averages for the groups are appreciably different. As statistically tested by use of Student's "t" test, the difference between the averages is very significant. The difference in the curative periods at the two temperatures is sufficient to indicate a difference of approximately 20% in dose levels under normal test conditions.

In another experiment ten animals were carried through four successive curative periods, maintained at 78° F. during the first, at 90° F. during the second, and at 78° F. for the third and fourth periods. Doses of 6 µg. of thiamine were administered for the first and third periods while 3-µg. doses were given the animals at the beginning of their second and fourth periods. In table 2 it will be seen that the average lengths of the first and the third curative periods induced by 6 µg. are quite similar, 9.1 and 9.9 days, respectively, while the average length of the second period, the response to a 3-µg. dose of thiamine in the 90° F. room, is 11.6 or greater than that of the first or third periods. In the fourth period where the animals were kept at 78° F. and received 3-µg. doses there were no cures of polyneuritis in four animals and the duration of cure was from 2 to 4 days for the others. This response is typical for this dose and does not reflect a failure of the rat to respond owing to repeated occurrence of polyneuritis. Since it has been adequately demonstrated that at a given temperature, over a wide range of thiamine dosage, the duration of curative response is proportional to the dose administered (Kline, Tolle and Nelson, '38), and since the duration of cure with 3 µg. at 90° F. is greater than with 6 µg. at 78° F. the data presented are interpreted to indicate a 50% reduction in thiamine requirement for the rat under the environmental conditions described.

TABLE 2

Successive curative periods with different environmental temperatures and thiamine dosages.

RAT NO.	FIRST PERIOD 78° F. 6γ B_1	SECOND PERIOD 90° F. 3γ B_1	THIRD PERIOD 78° F. 6γ B_1	FOURTH PERIOD 78° F. 3γ B_1
	days	*days*	*days*	*days*
3936	7	11	7	3
3938	7	11	8	No cure
3973	7	14	8	4
3985	14	13	19	3
3987	11	11	7	2
3988	12	14	11	No cure
4016	10	15	15	No cure
4040	7	9	5	2
4042	7	7	9	No cure
4043-A	9	11	10	3
Average	9.1	11.6	9.9	

Studies involving rat growth

The animals used for the growth study were weaned from mothers on an adequate stock diet, at a weight of 40 to 50 gm. and were fed the U.S.P. thiamine-deficient diet ad libitum. They were kept at 78° F. in litter cages on wire-screen bottoms until records of body weight, taken as specified in the A.O.A.C. procedure (Kline, Hall and Morgan, '41) for the growth assay of thiamine, indicated the end of the depletion period. This condition is reached on the day when the body weight of the animal is equal to or less than that on the fifth preceding day. The animals were then placed in individual cages, grouped as desired, and records of body weight and food consumption were taken twice a week. In all instances where thiamine intake was controlled, the desired dose of the U.S.P. Thiamine Hydrochloride Reference Standard was administered daily by stomach tube.

In a preliminary study two groups of twelve animals that had been depleted were maintained at 78° and 90° F. and fed 2.5 µg. of thiamine daily for 33 days. The dose was then increased to 10 µg. daily for 10 days and further to 100 µg. daily for 7 days. Both groups were then kept for 7 days at 78° F. with no supplement. Results are given in table 3. Greater gains in weight were made during the 33-day period by the animals at the higher temperature, even though their food consumption was lower. With an increase in thiamine intake to a near optimum level or to a level ten times the optimum, this situation was reversed, and gain in weight and food intake were greater at 78° F. During the last period when both groups of animals were maintained at 78° F. without thiamine, the food consumption of those removed from the higher temperature increased markedly.

TABLE 3

Weight gains and food consumption of rats at indicated temperatures and thiamine intakes.

DAILY SUPPLEMENT	AVERAGE DAILY WEIGHT GAINS		AVERAGE DAILY FOOD INTAKE	
	78° F.	90° F.	78° F.	90° F.
µg.	*gm.*	*gm.*	*gm.*	*gm.*
2.5 (33 days)	0.7	1.0	7.0	6.0
10 (10 days)	4.0	3.1	13.3	8.6
100 (7 days)	4.1	2.6	14.0	8.5
None — Both groups at 78°F. (7 days)	3.0	2.7	14.0	10.7

In another study forty-eight depleted animals were divided into six comparable groups, three of which were kept in the room maintained at 78° F. and the remaining three groups transferred to a room maintained at 90° F. One group in each room was fed 2.5 µg. of thiamine daily; a second group in each room received 10 µg. daily; and the remaining groups were changed to the diet that is used for the laboratory stock colony. Records were taken twice weekly of body weight and food consumption for a period of 5 weeks for all animals, and for 10 weeks for the groups receiving the lower level of thiamine. Results of the 5-week experimental period are given in table 4, and the growth rates of the two groups that received the lower level of thiamine for 10 weeks are illustrated in figure 1.

Rates of growth are expressed as average daily weight gains in grams — for simplicity of comparison, and also in conjunction with average daily food intake, to allow convenient calculation of the amount of gain per gram of food consumed. In the groups that received 2.5 µg. of thiamine daily, a suboptimal level, the animals at the higher temperature were able to make greater gains with lower food intakes, and this occurred during a period of uniform weight increase, before there was evidence of loss of weight in any individual, or of occurrence of polyneuritis. The superiority of utilization of food for growth is reflected in the grams gain per gram of food intake, the difference for these two groups being 0.072 gm. in favor of the animals at the higher temperature. For those animals that received 10 µg. of thiamine per day, the greater growth occurred in the lower temperature, since thiamine no longer was the limiting factor. Owing to the reduced food intake at the higher temperature, however, the amount of gain for each gram of food consumed was still in favor of the higher temperature, with a difference of 0.056 gm. Although growth on the diet of natural foodstuffs was improved, the relationships were much the same as for the intermediate groups, and the difference of gain per unit of food intake was 0.060 gm.

TABLE 4

Comparison of weight gains and food consumption of rats at indicated temperatures and thiamine levels.

(Experimental period — 35 days)

	B_1 DEFICIENT DIET PLUS 2.5 MICROGRAMS B_1 DAILY		B_1 DEFICIENT DIET PLUS 10 MICROGRAMS B_1 DAILY		STOCK DIET WITH NO ADDED B_1	
	78° F.	90° F.	78° F.	90° F.	78° F.	90° F.
Av. daily weight gains (gm.)	0.8	1.1	3.5	3.0	5.1	4.6
Av. daily food intake (gm.)	7.0	5.6	12.7	9.0	19.0	12.6
Grams gain per gram food intake	0.124	0.196	0.275	0.333	0.304	0.364

In the two groups of animals given the lower level of thiamine and maintained throughout a 10-week period growth increased uniformly at the higher temperature with no evidence of polyneuritis. For the animals maintained at 78°F., the growth curve shows a plateau after the seventh week, and four of the eight animals died of polyneuritis before the end of the experimental period.

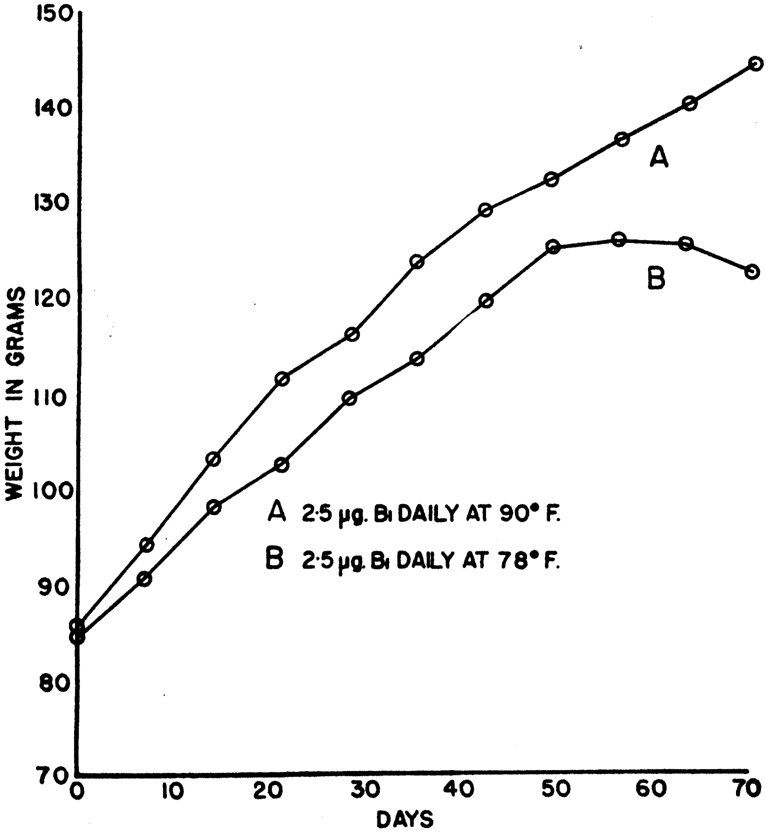

Fig. 1 Growth curves on groups of rats at 78° and 90°F. fed 2.5 µg. of thiamine daily.

DISCUSSION

From results of the rat-curative experiments presented it would seem logical to conclude that as environmental temperature of the rat is increased, the daily requirement for thiamine is reduced. Such a conclusion is in keeping with what is known about the function of thiamine in the utilization of food for energy. With increased environmental temperature the amount of energy needed for maintenance of body temperature is decreased. Drill and Shaffer ('42) have concluded that the increase in energy expended in experimental hyperthyroidism caused an increased requirement for vitamin B_1 and other vitamins of yeast.

In the growth studies described, thiamine intake and environmental temperature were the two important factors affecting growth response and food consumption. In the animals maintained at the higher environmental temperature consumption of food was lower than that of control animals, owing undoubtedly to a reduced metabolic rate. A depressing effect of increased environmental temperature upon metabolic rate has been pointed out by Horst, Mendel and Benedict ('30) and has been discussed more recently by Herrington ('40). A direct relationship between growth rate and rate of metabolism has been demonstrated by Kibler and Brody ('42). Under the conditions of our experiments with optimal thiamine intake food consumption and growth response were directly affected by environmental temperature, and thus could not be related to thiamine requirement. For a clear demonstration of the effect of temperature upon thiamine requirement it is essential to use a suboptimal thiamine level, where the animal is sensitive to variations in factors affecting thiamine requirement. Sarrett and Perlzweig ('43) although obtaining, with paired feeding technic, higher growth rates in rats at elevated temperatures, were unable to demonstrate an effect of environmental temperature on thiamine requirement owing undoubtedly to the feeding of adequate levels of thiamine.

Mills ('41) has reached the conclusion from studies similar to ours that thiamine requirement of the rat is increased with increased environmental temperature. It should be pointed out that in his experiments, since thiamine supplements were mixed with the diet, thiamine intake for each animal varied with food intake, and was, in effect, controlled by environmental temperature. If actual amounts of thiamine ingested are calculated for these groups of animals that received suboptimal intakes of thiamine, it will be noted that the animals kept at the higher temperature made greater weight gains on less thiamine than the animals at the lower temperature. This relationship is similar to that found in our experiments in which thiamine, fed as a separate supplement, was independent of food intake.

It is to be expected that a diet, adequate in respect to thiamine, when consumed in a temperate climate, would also be satisfactory under tropical conditions, even though a reduced food intake resulted from the higher environmental temperature.

In experiments similar to those reported here, Edison and Molitor ('43) have concluded that thiamine requirements in the tropics are not greater than in temperate climates, but on the contrary are lower, owing to decreased activity of the animal.

SUMMARY

Evidence has been obtained in studies on rats involving both cure of polyneuritis and growth, which shows that an increase in environmental temperature results in a decreased thiamine requirement. It appears that this decrease approximates the decrease in caloric requirement at the elevated temperature. Maintenance of a uniform environmental temperature is essential for precision in performing the rat-curative assay for thiamine.

ACKNOWLEDGMENT

The authors wish to thank Lila F. Knudsen for her helpful suggestions and statistical analysis.

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THE RELATION OF THE DIETARY CA:P RATIO TO SERUM CA AND TO PARATHYROID VOLUME

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THREE FIGURES

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It has been shown by a number of investigations (Ham et al., '40; Carnes et al., '42) that various conditions associated with hypocalcemia or hyperphosphatemia, or both, lead to hyperplasia of the parathyroid glands. Evidence has also been given that parathyroid enlargement in such instances may be associated with increased secretory activity of the glands (Baumann and Sprinson, '39). The changes in the levels of blood Ca and P in these instances are usually reciprocal and the question has arisen whether the parathyroid enlargement is primarily related to the lowering of the blood Ca or to the increase in blood PO_4.

Ham and coworkers ('40), by adding phosphate to a stock diet, were able to obtain a small increase in the serum PO_4 of rats without changing the serum Ca. The parathyroids of these animals failed to enlarge. The glands did enlarge in rats on a Steenbock low Ca diet with low serum Ca values. It was concluded that hypocalcemia and not hyperphosphatemia is the stimulus to parathyroid enlargement.

In contrast to this, on adding graduated amounts of P to the Steenbock low P diet, Carnes and coworkers ('42) obtained stepwise increases in parathyroid volume in younger rats associated with rising serum PO_4 levels even in the absence of hypocalcemia. However at both extremes of the scale of dietary Ca/P ratio, the serum calcium changes were significant and the serum PO_4 values not always consistently altered. Further experience has cast doubt on the reliability of data obtained with the Steenbock diet which is inadequate in several respects and produces a very poor general condition of the experimental animals. Differences in the basal diet, form and quantity of added PO_4, and age of the experimental animals may have contributed to the discrepancies between the results of these two groups.

Recently Patt and Luckhardt ('42) concluded from a series of experiments on dogs that a low blood calcium is a direct stimulus for the parathyroid glands to produce more hormone. They lowered the blood calcium in their animals by injecting oxalate and also perfused decalcified blood through thyroid-parathyroid preparations, obtaining what they believed to be evidence for increased parathyroid activity. Possible alterations in blood PO_4, brought about by the procedures, were not excluded in these experiments.

The changes in Ca and PO_4 levels of the blood in response to variations in dietary Ca and P have been thoroughly studied by Shohl and Wolbach ('36) in an investigation of rickets in rats. They confirmed the findings of Kramer and Howland ('32) and of Bethke et al. ('32) that the blood levels of Ca and PO_4 reflect the dietary Ca/P ratio. Lowering this ratio decreased the serum Ca and increased the serum PO_4. Raising the ratio had the opposite effect on the serum values. They also found that, with a constant dietary Ca/P ratio, raising the absolute amounts of Ca and P in the diet produced a corresponding rise in both the serum Ca and serum PO_4.

If the blood level of either Ca or PO_4 is the primary stimulus of the parathyroid gland, it should be possible to determine which of these two factors is the essential one by experimentally altering the dietary intake of Ca and P independently over a wide range and by following the consequent changes in the blood levels and parathyroid volume. This can be done with a minimum of interference with the general nutrition in adult rats on a good basal ration.

EXPERIMENTS

Male albino rats, in most instances litter-mates, weaned at the age of 4 weeks and placed on our stock diet[1] were selected from the stock colony at 10 weeks of age. They were fed for 4 weeks a diet[2] devised by Zucker and Berg ('43) which is adequate in every essential except Ca and P and is thoroughly freed of vitamin D. This diet favors optimal growth (Zucker and Zucker, '42) if supplemented with proper amounts and Ca and P. The desired amounts of Ca and P were added in the form of $CaCO_3$ and KH_2PO_4. After 4 weeks the animals were anesthetized with ether and blood was drawn from the right auricle. Individual analyses were made for Ca (Clark and Collip, '25) and inorganic

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[1] Rockland rat diet (D-free).

[2] Heated egg albumen (E) or alcohol extracted beef fibrin (F), 20%; modified Wesson salt mixture, 1.2%; Wesson oil, containing 5% carotene in oil, 2.0%; rice bran extract, 10%; cane sugar, to make 100%.

P (Kuttner and Cohen, '27) on fresh samples of serum. The parathyroid glands were fixed in Bouin's fluid, imbedded in paraffin, serially sectioned at 10 micra, and the volumes determined by tracings and planimeter measurements. Bones were sectioned and studied histologically and the bone ash was determined on the right femur in some of the groups.[3] Serum protein estimations were made in some groups by the falling drop method.

TABLE 1

Chemical and anatomical data on 14-week-old rats following 4-week feeding of diets of differing Ca and P content.

GROUP	DIET	% DIETARY		DIETARY CA/P RATIO	BODY WEIGHT (GM.)		PARA-THYROID VOLUME (MM.^{3})	SERUM (MG. %)		SERUM CA/PO$_4$ RATIO	BONE ASH %	NO. RATS
		CA	P		Initial	Final		CA	PO$_4$			
1	F–908–A	1.053	.07	15.1	197	246	.173	11.9	3.4	3.5	60.2[1]	10
2	E–908	.615	.07	8.8	194	225	.181	11.6	2.5	4.6	..[1]	5
3	F–918–A	2.053	1.17	1.8	163	239	.311	10.3	7.3	1.4	..	9
4	F–918	1.053	.62	1.7	164	248	.313	10.4	8.2	1.3	..	8
5	F–918	1.053	.62	1.7	201	269	.283	11.1	7.1	1.6	64.0	6
6	E–918	1.015	.62	1.6	199	251	.348	10.6	..	..	62.4	6
7	E–907	.615	.47	1.3	178	241	.369	11.0	..	..	63.1	5
8	F–913	.053	.07	.8	207	269	.371	9.6	4.6	2.1	61.0[1]	6
9	F–909	.053	.47	.11	202	252	.475	8.1	7.3	1.1	56.0[1]	5
10	E–909	.015	.47	.03	178	188	.508	7.7	..	..	59.2[1]	6
11	E–909	.015	.47	.03	212	223	.551	7.3	..	..	.	6

[1] Rickets present.

RESULTS AND DISCUSSION

The complete data are given in table 1. By adding either Ca or P, or both simultaneously to the basic diet, nine variations of the dietary Ca/P ratio were obtained ranging from 15 to 0.032. The absolute level of dietary Ca varied from approximately 2% to 0.015% and the P from approximately 1.2% to 0.07%. Because of the age of the animals and the relatively short duration of the experiment, growth was only moderately retarded on low Ca and low P variations of the diet. Both types of rickets were mild as judged histologically and by the bone ash values.

On the basis of parathyroid size the animals fall into three distinct divisions. The glands were very small when the dietary Ca/P ratio was far above optimal (groups 1 and 2). The glands were very large when this ratio was far below optimal (groups 9–11). When the dietary

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[3] We are indebted to Miss Margaret Young for the bone ash determination.

Ca/P ratio approached the optimal range (9), even though the absolute quantities of Ca and P were varied over an extremely wide range, the glands were intermediate in size and the groups did not differ significantly between each other (groups 3–8). Figure 1 illustrates the interpendence of parathyroid volume and the per cent of dietary Ca and P. Figure 2a shows, incidentally, that the relationship between parathyroid volume and the logarithm of the dietary Ca/P ratio closely approximates a straight line.

The effect of the diets on serum Ca level was inverse to their effect on the parathyroid volume. The table shows that on the basis of serum values the animals fall into the same three divisions that were made on the basis of parathyroid size. On a diet of high Ca/P ratio there was a mild hypercalcemia (groups 1 and 2). On a diet of very low Ca/P ratio there was a moderate hypocalcemia (groups 9–11). On a diet with a Ca/P ratio approaching the optimal, the serum Ca fell within the accepted range of normal, even though the absolute quantity of dietary Ca was varied within very wide limits (groups 3–8). Figure 2b shows that the relationship between the serum Ca level and the logarithm of the dietary Ca/P ratio also approximates a straight line. Under the conditions of our experiment the serum Ca level was not proportional to the per cent of dietary Ca at a constant Ca/P ratio. The serum Ca was not significantly different on diets of 2, 1, and 0.6% Ca, respectively (groups 3–7) and it was not greatly depressed with as little as 0.053% dietary Ca (group 8) when the dietary Ca/P ratio remained in the neighborhood of 1. These findings are confirmed by unpublished data on a sufficient number of animals which at weaning (age 4 weeks) were placed on the experimental diets for 4 weeks and then killed.

The serum phosphate level has been found, in agreement with Shohl and Wolbach ('36), to depend on both the dietary Ca/P ratio and the absolute quantity of dietary P up to a certain maximal level of serum PO_4. This maximum is apparently dependent upon factors other than dietary Ca and P and is also a function of the age of the animal. It was approximately 7–8 mg. % in this experiment but in immature rats it is about 10–12 mg. %. The level of serum PO_4 thus does not bear a simple inverse relationship to the serum Ca level.

Figure 3 shows that there is a fairly close inverse proportionality between the parathyroid size and the level of serum Ca over the entire range investigated. This relationship deviates somewhat from a straight line, perhaps due to an inherent limitation of maximal para-

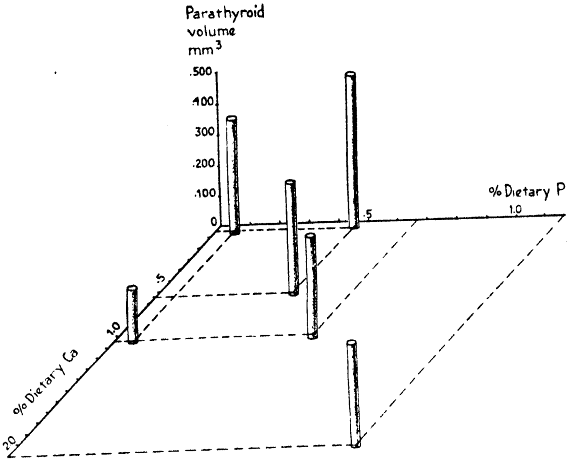
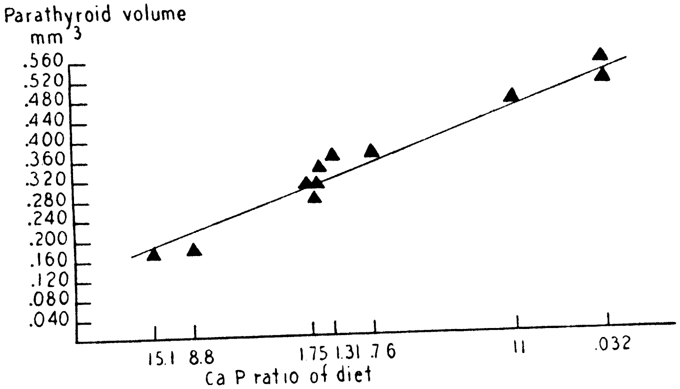


Fig. 1 Relationship between the per cent dietary calcium, the per cent dietary phosphorus, and the parathyroid volume.



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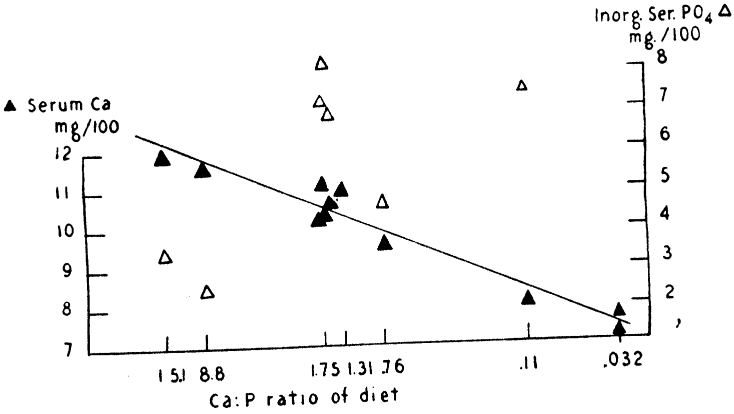

b

Fig. 2a Relationship between the parathyroid volume and the logarithm of the Ca/P ratio of the diet.

Fig. 2b Relationship between the serum calcium concentration and the logarithm of the Ca/P ratio of the diet.

thyroid growth in the hypocalcemic groups within the period of 4 weeks. The same deviation in the line could be due to a limitation, by factors other than the diet, in the maximal serum Ca attainable. The poorest alignment of the points occurs in the midportion of the curve within the normal range of serum Ca. This scattering of the points is not eliminated by expressing the parathyroid volume per unit of body weight.

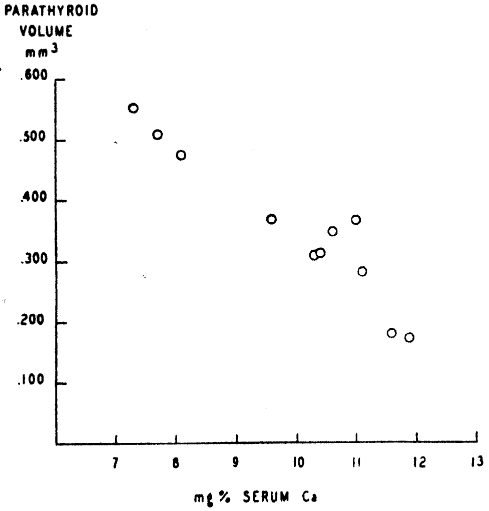

Fig. 3 Relationship between the serum calcium concentration and the volume of the parathyroid glands.

Table 1 shows that the relationship between parathyroid size and serum PO_4 level is not a close one over the entire range, due to the low maximal level of serum PO_4 attainable. The apparent dependence of parathyroid volume on serum PO_4 concentration in younger animals was possibly due to the much higher maximum attainable at that age (Carnes et al., '42). Under the present conditions no such dependence is apparent.

It has been suggested, on the basis of considerable circumstantial evidence that the stimulus for the parathyroid glands to produce more hormone is a serum Ca ion level below normal (Albright, '42). The chief factor influencing the ionization of serum Ca is the serum protein concentration (McLean and Hastings, '35). The serum protein was not determined in all the groups of this experiment but in groups 6, 7, and 10 (diets 918, 907, and 909) the average value was 6.6, 6.6, and 6.8%, respectively, and in unpublished experiments on rats of different ages on diets 908 and 913, no significant deviations in serum protein concentration have been encountered in well-nourished animals. It is unlikely that the serum PO_4 concentration influences the ionization of serum Ca under the present experimental conditions. McLean and Hastings ('35) found no evidence of a non-ionized Ca-PO_4 complex in the plasma under a variety of experimental and spontaneous pathological conditions. Such a complex is producible in the blood under certain conditions, however (McLean and Hinrichs, '38) and the development of manifest tetany in the parathyroidectomized dog has been said to be closely correlated with a critically low ratio of serum Ca/serum PO_4 (Reed et al., '28). It has also been suggested that the parathyroid hyperplasia in renal disease is a result of a disturbance in the Ca/P ratio of the plasma (Jaffee and Bodansky, '43). The table contains the serum Ca/PO_4 ratios of most of the groups in the present experiment. When the various suboptimal diets (groups 1, 2, 8, 9) are considered, a rather close inverse proportionality is observed between this ratio and the parathyroid volume. But this is no closer relationship than that between the serum Ca and parathyroid size in the same groups and the alignment of these groups with those on the more nearly optimal diets (groups 3, 4, 5) is poor. Moreover, the difference in serum Ca/PO_4 ratio between some of the animals with greatly enlarged parathyroids and those with normal-sized glands is not great enough to be significant.

Whereas the present experiment does not yield a decisive answer to the fundamental question of what constitutes the physiological stimulus to parathyroid activity, the data do indicate that over a wide range, both above and below the normal, there is a close correlation between the total serum Ca concentration and parathyroid volume under closely controlled conditions. The possibility remains that the Ca ion concentration is the important moiety. These results are essentially in accord with those of Ham and associates ('40) who concluded that hypocalcemia is the stimulus to physiological hypertrophy of the parathyroid glands. It may be anticipated that other factors, especially the specific growth essentials, will influence the translation of this stimulus into anatomical enlargement. The failure to obtain high enough values of serum PO_4 in this experiment prevents any further conclusions on the possible influence of serum PO_4 level on the size of the glands under other circumstances.

CONCLUSIONS

A close direct proportionality has been found between the logarithm of the dietary Ca/P ratio and the serum Ca concentration in mature rats on an adequate basal ration over a very wide range of dietary Ca and P. A similarly close inverse proportionality has been found between the logarithm of the dietary Ca/P ratio and the volume of the parathyroid glands. The parathyroid volume and serum Ca concentration accordingly varied inversely and with a nearly linear relationship between serum Ca levels of 7.3 and 11.9 mg. %. The serum inorganic PO_4 level was less uniformly related to the parathyroid size under the present experimental conditions.

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SOME FACTORS INFLUENCING THE FECAL ELIMINATION OF THIAMINE BY HUMAN SUBJECTS

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TWO FIGURES

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Studies of thiamine metabolism in human subjects have, for the most part, been focused upon data obtained from measurement of the urinary excretion, plasma level, or tissue concentration of the vitamin. Where determinations of fecal thiamine have been made, it has often been difficult to interpret the results in the light of the conditions of the experiment, and little significance has been attached to any relationship between the fecal thiamine output and the state of thiamine nutrition of the individual.

Although the regular incidence of thiamine deficiency symptoms in the absence of dietary sources of thiamine has been reported, recent evidence presented by Najjar and Holt ('43) shows that, in their experience, incidence of deficiency symptoms on a diet low in thiamine was not universal. Subjects who did not show clinical signs of deficiency following a prolonged period of the extremely low thiamine intake furnished by a purified diet appeared to eliminate a larger proportion of "free" thiamine in the feces, as contrasted to the "bound" thiamine released by enzymatic digestion, than did the subjects in whom the deficiency could be recognized. The possibility that thiamine from the body was being excreted into the intestinal tract was explored through the injection of massive doses of the vitamin and ruled out when no increase in the fecal thiamine output resulted. These workers believed that the subjects free from clinical deficiency symptoms were able to utilize the synthesized thiamine and that the diet might well be a determining factor in establishing thiamine requirements.

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The effect of the diet upon the synthesis of nutritional factors in the intestinal tract has been recognized since the first demonstration of the phenomenon of refection by Fredericia in 1926. Certain raw starches and dextrin have been found to be most effective in stimulating thiamine synthesis but there is no conclusive evidence known to the authors to show that the rat is able to use this synthesized vitamin if coprophagy is prevented. It has more recently been shown that certain factors resulting from microbiological activity in the tract such as vitamin K and biotin are available to the body. It is possible that thiamine, too, under certain conditions, may be absorbed from the lower portions of the digestive tract.

It is evident that clarification of the role of intestinal synthesis in man requires further study of the diet as a factor in stimulating the synthesis of available thiamine. The fecal thiamine elimination of several groups of subjects on a modified milk diet has been studied in this laboratory; results will be discussed and compared with the observations of other workers.

EXPERIMENTAL

It should be stated that the series of experimental diets, from which these results were obtained, was planned with the primary aim of studying various factors influencing gastro-intestinal motility in connection with another study (Stettler, '44). They are equally valid, however, in providing data for a consideration of some of the factors which appeared to influence the thiamine content of the feces.

The low fiber basal diet used was composed of milk, butter, eggs, ice cream, Cheddar cheese and unenriched white bread and supplies about 0.6 mg. of thiamine.[2]

Subjects were graduate and senior women in Home Economics at the University of Wisconsin; that they were satisfactory subjects for this study was indicated by the uniformity of response of urinary thiamine excretions during the first days of the period of controlled level of thiamine intake.

Group 1 consisted of twelve subjects who were given supplements of 600 gm. of drained crushed pineapple or 600 ml. of pineapple juice in addition to the basal diet in order to determine the effect of fiber content of the diet upon thiamine absorption and fecal thiamine elimination. For six of the subjects, the 7-day fiber period preceded the juice period of the same duration; the reverse sequence was followed by the other

[2] Composition: milk — 810, egg — 50, ice cream — 100, Cheddar cheese — 50, bread (white, unenriched) — 125, and butter — 30 gm.

six. Inasmuch as the pineapple juice was obtained by draining the crushed pineapple, the content of soluble constituents was presumably similar (both juice and fiber were found to contain the same concentration of the thiamine); hence, the fiber in the diet was the principal difference between the two periods. Basal diet and pineapple supplements supplied a thiamine intake of 1.3 mg./da.

In the studies with subjects of group 2, an entirely different type of diet, based on meat, was compared to this same modified milk basal in two 5-day periods. The milk, eggs, and cheese of the basal diet of the first period were replaced, during the second, by 300 gm. of ground cured ham[3]; whole crushed pineapple, bread, butter and ice cream were included in both. Although the ham diet supplied appreciably more thiamine, 3.2 as contrasted with 1.3 mg./da., certain comparisons could be made.

Subjects of group 3 were given thiamine chloride supplements of 2 to 10 mg. for 30- to 60-day intervals between two test periods during which the basal diet with supplements of pineapple juice supplying 0.3 mg. thiamine was given so that the total thiamine intake was about 1.0 mg.

Fecal collections were separated by means of carmine markers and daily 24-hour urine collections were made. Ten per cent food aliquots were assayed periodically for thiamine. All determinations were made by means of the thiochrome method, essentially that of Hennessey ('42).

RESULTS

That the substitution of crushed pineapple for pineapple juice in the diet had a marked effect upon the thiamine content of the feces may be seen from table 1. In nine of the twelve subjects, irrespective of sequence of periods, the fecal thiamine was significantly increased above that on the juice period when the coarse pineapple fiber was fed (table 1). Although this is in agreement with the observation of Knott and Schlutz ('39) that the low fiber milk diet produced a lower fecal thiamine content than did the diet to which cereal roughage was added, the lack of variation in the urinary thiamine excretion does not tend to support her hypothesis.

The urinary thiamine excretions of the subjects did not vary significantly with respect to individual subject, to fiber content of the diet, or to sequence of periods as may be seen from table 1. Inasmuch as more than one-half of the total thiamine intake was supplied by the

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[3] Supplied through the courtesy of Professor Elvehjem from experimental material in his laboratory.

crushed pineapple and juice supplements to the basal, it would seem that, if interference of the fiber residues with absorption were present, it would be reflected in the level of urinary thiamine excretion. **Despite the fact that much of the stool voided during the ingestion of the fiber appeared to contain large quantities of apparently intact pineapple tissue, no consistent trend toward lowered urinary thiamine excretion** could be traced. There was no apparent correlation in most instances between the urinary and fecal thiamine eliminations (table 1); even in the case of subjects D. R., where the fecal thiamine output was as low as 8 µg./da., and K. O., who reached a 902 µg. level during the fiber period, urinary thiamine levels were remarkably similar, 206 and 200 µg./da, respectively. Sequence of periods appeared to have no definite influence thus giving validity to the use of short periods such as these, often necessary in human dietary studies.

TABLE 1

Urinary and fecal thiamine eliminations of twelve subjects of group 1 on a basal diet with low and high fiber supplements.

SUBJECT	THIAMINE				SUBJECT	THIAMINE			
	Urinary when diet contained pineapple		Fecal when diet contained pineapple			Urinary when diet contained pineapple		Fecal when diet contained pineapple	
	drained crushed	juice	drained crushed	juice		drained crushed	juice	drained crushed	juice
	µg./day		*µg./day*			*µg./day*		*µg./day*	
When fiber period preceded the juice period					When juice period preceded the fiber period				
DR	204	208	9	9	MS	300	259	351	186
KO	195	203	902	337	CL	234	263	476	309
RB	263	230	484	370	GJ	299	210	615	252
EB	196	299	341	241	EL	250	316	332	298
SL	285	300	40	74	CM	247	260	246	339
HW	200	240	514	92	EO	231	244	336	221
Av.	224	247	381	187	Av.	260	259	393	267
					Grand average	242	253	387	227

It was thought that various data collected during the course of laxation studies carried out simultaneously might be of significance, inasmuch as the character and amount of fecal material is believed to be related to the kind and activity of the intestinal flora. On the basis of dry weight of feces, the residues of bacteria account for a large proportion of the fecal mass; likewise the fate of a complex carbohydrate ingested, such as cellulose, may, in part, be determined by the intestinal flora. Data were examined to see whether correlations existed between the daily fecal thiamine elimination and such factors as the bulk of feces on the basis of dry weight, moisture content, time for passage of carmine marker and possible individual variations between subjects.

Figure 1 illustrates the relationship between the total thiamine content of the feces per day, dry weight of the fecal material, and the concentration of thiamine per gram of dried feces for both fiber and juice periods. From this series arranged in ascending order of fecal thiamine, it may be seen that the trend of larger fecal outputs parallels, in general, the increase in fecal weight, the concentration of thiamine remaining relatively constant. Unusually high thiamine output appeared to be associated with greater bulk of feces. This was found to be true for rats by Leong ('37) and, inasmuch as the bulk of the feces is considered to be determined largely by numbers of bacteria, the thiamine content of the feces may be inferred to be related to numbers and activity of organisms in the tract synthesizing thiamine.

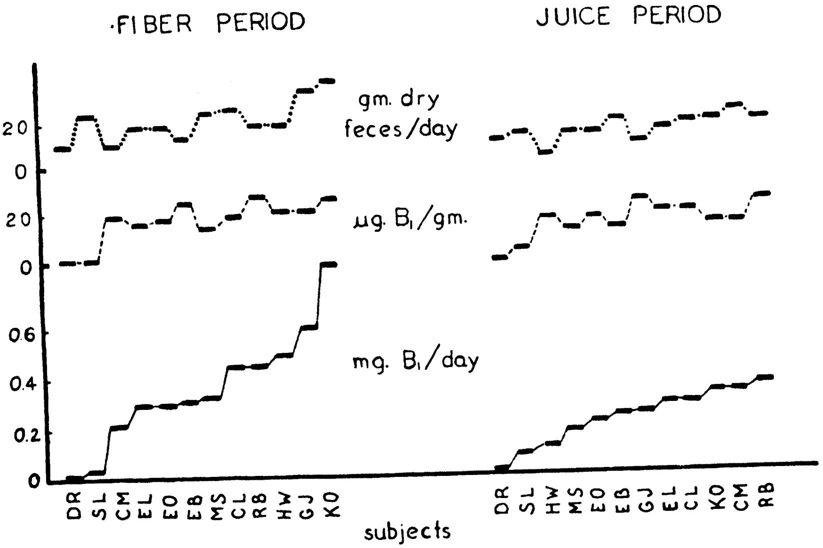

Fig. 1 Relationships between fecal thiamine elimination, dry weight of feces, and concentration of fecal thiamine on fiber and juice supplemented periods.

It has been shown (Stettler, '44) that the tendency of the crushed pineapple additions to the diet to increase the bulk of the feces on the basis of dry weight was far greater than could be accounted for by the actual amount of fiber ingested during this period as contrasted to the juice period. Thus the pulp appeared to stimulate bacterial activity and, simultaneously, the fecal thiamine content.

That the additional thiamine found under these circumstances was a result of bacterial activity, rather than incomplete absorption higher in the tract, was further indicated by calculations based on the possible thiamine content of an amount of pineapple equal to the difference in weight of feces as voided between crushed pineapple and juice periods. The added thiamine found in the feces on the fiber period could not be accounted for satisfactorily by the amount of thiamine that would be contained in pineapple passing through the tract.

Walker and Nelson ('33) have shown that, of a number of plant cells tested, intact yeast cells alone retained significant amounts of thiamine not available for absorption when fed fresh without heating.

The concentration of fecal thiamine varied from 13 to 26 μg./gm. in most instances (fig. 1) and is unusual only in two subjects, D. R. and S. L., for whom the range was 0.8 to 5.7 μg./gm. It may be seen that the amount of feces on the dry weight basis was not exceptionally low in either case (fig. 1) so that the extremely low total thiamine output resulted from the low concentration.

Of a total of some twenty-five subjects for whom determinations of fecal thiamine elimination have been made in this series in connection with various studies, five have been characterized by extremely low levels of thiamine in the feces. Even during periods of ingestion of certain types of fresh yeast, ordinarily tending to elevate greatly the fecal thiamine content, two subjects maintained consistently low levels. Three of the five have taken part in several diet studies and in all test periods retained this same uniqueness of response. In these individuals, some special condition apparently existed in the digestive tract which resulted in this particular effect, possibly the destruction of thiamine by organisms or other influences prevailing in the tract itself.

It might be thought that the slower passage of the intestinal contents with an accompanying increase in water absorption would lower the moist fecal weight and possibly the thiamine content of the feces. Comparison between lengths of time for carmine passage and relative fecal moisture and thiamine content indicated, however, that no consistent relationship between these factors occurred in the twelve subjects. It should be noted in this connection, however, that fluoroscopic studies have shown that the time for passage of a carmine marker is not necessarily indicative of the speed of passage of material through any particular portion of the tract.

In general, it is concluded that the influence of the crushed pineapple in elevating the thiamine content of the feces is through the stimulation of intestinal synthesis. Large amounts of water, sugars, and other water soluble constituents held in the tract, in addition to the cellulose, would, presumably, create a favorable medium and would tend to encourage growth of microorganisms.

In the above experiments, a relatively limited diet with additions of known factors such as pineapple fiber has been used. It is of interest to compare the results with those obtained from an entirely different type of diet. After a preliminary 5-day period on the pineapple supplemented milk basal, subjects of group 2 were given the modified milk diet with equal amounts of crushed pineapple and juice. Immediately thereafter, 300 gm. of ground cured ham was substituted for the milk, eggs and cheese of this diet for another 5-day period.

TABLE 2

Average daily urinary and fecal thiamine outputs of four subjects of group 2 on milk and meat basal diets.

	URINARY THIAMINE		FECAL THIAMINE	
	Milk basal[1]	Meat basal[2]	Milk basal[1]	Meat basal[2]
Amount	272 μg.	759 μg.	341 μg.	312 μg.
Percentage of intake	19%	24%	24%	10%

[1] 1.4 mg. intake.

[2] 3.2 mg. intake.

Despite the higher thiamine intake during the meat period (3.2 as contrasted with 1.4 mg. on the milk period) the absolute amount of thiamine in the feces was not increased above that found during the preceding period. It has been commonly thought that meat influences markedly the character of the intestinal flora; if this was the case in this instance, it does not appear that this change affected the fecal thiamine content (table 2). Any influence of the unusually high salt content (10 gm.) of the diet could not be evaluated. The percentage of the thiamine intake excreted in the urine at this level of intake was within the expected range.

Although in nine of the twelve subjects of group 1 variations in fecal thiamine output between fiber and juice periods were inversely related to the urinary thiamine excretions, three subjects did show a striking relationship between urinary and fecal thiamine outputs in response to the crushed pineapple additions to the diet (fig. 2). Subjects G. J., M. S. and R. B. exhibited higher urinary thiamine excretions during the fiber period; the increments in fecal and urinary thiamine for this period over those of the juice period were roughly proportional. However, amounts of total fecal thiamine for these subjects were no greater than for subjects K. O. and H. W. who had excreted lower amounts of urinary thiamine on the crushed pineapple period despite the high fecal output. Following the procedure of Najjar and Holt ('43), determinations of free thiamine were made in the feces of subjects G. J. and K. O., whose fecal thiamine outputs had been markedly increased by the inclusion of pineapple fiber in the diet but who differed in response of urinary thiamine excretion. In both subjects, it was found that the free thiamine constituted about 50% of the total and that the difference between fiber and juice periods was no greater for G. J. than for K. O. This leaves questionable the relation of this work to the interpretation of Najjar and Holt that some subjects were able to utilize thiamine synthesized in the digestive tract.

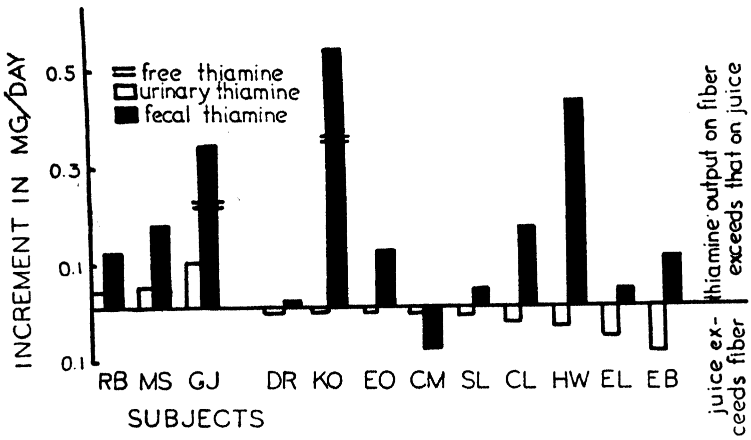

Fig. 2 Degree to which a supplement of crushed pineapple or pineapple juice is associated with an excess of urinary or fecal thiamine. It is to be noted that there is little apparent correlation between urinary and fecal thiamine in this respect.

SUMMARY

1. The ingestion of large amounts of plant fiber (pineapple) tended to be associated with relatively large fecal thiamine eliminations. Interference with absorption was not indicated so much as stimulation of intestinal synthesis of thiamine.

2. In general, no correlation was found between variations in urinary and fecal thiamine output for high and low fiber periods, even when there existed in the fecal output a difference great enough so that variations in urinary excretion might be reflected.

3. There were indications that in certain subjects there was destruction of considerable amounts of thiamine in the digestive tract.

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STUDIES ON THE CAROTENOID AND VITAMIN A LEVELS IN CATTLE

I. SEASONAL CHANGES OF THE CAROTENOID AND VITAMIN A LEVELS AND THE NORMAL CAROTENOID-VITAMIN A RATIO OF THE BLOOD

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TWO FIGURES

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INTRODUCTION

Various investigators have reported on the carotenoid and vitamin A content of the liver and blood of men and animals under different dietary and pathological conditions (Barron, '42; Boyer et al., '42; Braun and Carle, '43; Davis and Madsen, '41; Deuel et al., '42; Gallup and Kuhlman, '41; Guilbert and Hart, '34, '35; Keener et al., '42; Lindquist, '38; Ralli et al., '41; With, '40).[2] A few of these investigators, especially Stepp and Wendt ('37), have made an effort to correlate the carotenoid and vitamin A levels in human blood and were unable to obtain consistent results. This failure is probably due to the uncertain intake of vitamin A itself, which masks the results of such studies on human material. In cattle, if maintained without vitamin A supplement, this complicating factor is eliminated, since all the vitamin A present in the blood has been converted from carotenoids within the animal. Studies on cattle may thus contribute to a better knowledge of the normal ratio of carotenoids to vitamin A in the blood as well as in the liver.

Such information, as well as other data on vitamin A metabolism, have been obtained in an experiment which had been primarily designed to test the effect of dietary regimes upon the resistance to Brucella abortus infection in cattle.[3] During this experiment, nineteen different blood constituents of forty-nine cows maintained in four dietary groups with different carotenoid and vitamin A intake, were repeatedly tested for a period of 1 year, among them tests for carotenoid and vitamin A. This report will present a brief discussion of the seasonal changes observed for the carotenoid and vitamin A levels in the blood of these animals and an analysis of their carotenoid-vitamin A ratios.

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These studies were supported in part by grant from the U. S. Bureau of Animal Industry under cooperative agreement with the Regents of the University of California.

[2] For further references, see chapter on fat-soluble vitamins in "Annual Reviews of Biochemistry," published since 1932 by Annual Reviews, Inc., Stanford Univ.

[3] This main project has been conducted by Dr. C. M. Haring, Dr. J. Traum and the author.

EXPERIMENTAL ANIMALS

All animals were maintained on nonirrigated, natural grass pasture in Strawberry Canyon, Berkeley, California, without supplementary feed from March until September. After September 1, the animals were divided into four groups: (1) the pasture group (P) consisted of animals which remained on the same pasture without supplementary feed throughout the year; (2) a pasture group (PA) of animals which were maintained on the same pasture as the P group but were fed 400,000 I.U. of vitamin A in the form of shark-liver oil twice a week from September through November; (3) a group of animals which were transferred from pasture into the barn (B group) and was maintained there on a carotenoid and A-deficient diet consisting of beetpulp, cottonseed-meal and rolled barley; (4) a group of barn animals which received a shark-liver oil supplement (400,000 I.U. twice a week) from September through November (BA groups).

The animals varied in age from 1 to 13 years. The majority were Holstein cows, but there were also a few Jerseys, 1 Hereford, and a few cross-bred animals.

On October 2nd, the pregnant animals were exposed to Brucella abortus. Most of the nonvaccinated animals and a number of the vaccinated animals aborted during November and December. The infection with Brucella abortus had no effect on the blood constituents tested including the carotenoid and vitamin A levels, except those changes which were produced during subsequent abortions; therefore, the results obtained can be used for a general analysis of carotenoid and vitamin A changes.

METHODS

The carotenoid and vitamin A content of the blood was measured with the Cenco photometer, using the method described by Kimble ('39). Wratten filter no. 47 was used for the reading of carotenoids and Wratten filter no. 58 for the blue color developed after addition of $SbCl_3$ in chloroform to the prepared sample. Kimble's calculation for vitamin A and carotenoid corrections was used. A preparation of pure β-carotene was used for the standardization of the carotenoid readings. It is realized that the data for vitamin A, as expressed in I.U./ml. or I.U./gm. are probably only relative, but this does not affect the results of these comparative studies.

RESULTS AND DISCUSSION

Seasonal changes of the carotenoid and vitamin A levels

From March to September 1st all animals were on pasture and showed decreasing carotenoid levels in successive tests made during this period. A tendency for carotenoid levels of old animals (older than 1 year and 6 months) to remain at high levels longer than those of young animals (younger than 1 year and 6 months) was noted but this difference did not reach statistical significance in an analysis of variance (Snedecor, '34) based upon all data for carotenoid levels between March and September.[4] Differences between means of tests, i.e., seasonal changes, however, were found to be highly significant. A significant difference was also revealed to exist between the means of animals, i.e., individual differences, partly due to differences in breed of animals, which caused differences in maximum levels (= highest level found during 1 year of testing) and time at which the decrease of carotenoid levels started.

The rapid decrease of carotenoid levels lasted until August. Animals which remained on pasture after September 1st (P group) maintained low levels until November and showed a rapid increase in carotenoid levels after this date. Those animals which were maintained on the same pasture but received a vitamin A supplement (PA group) showed on the average lower carotenoid levels than the animals of the P group. This confirms observations of Deuel, Hallman and Mattson ('42) that feeding of vitamin A lowers the carotenoid level.

Animals of the B group continued to show a decrease of their blood carotenoid level until November, when no carotenoids could be detected in the blood by methods used here. Administration of vitamin A (BA group) showed no effect on these low levels.

As in the case of carotenoids, vitamin A levels dropped in pasture animals from about March until August, remained low until November, and increased after that date. An analysis of variance based on March-September data revealed no significant difference between age groups, highly significant differences between means of tests, i.e., seasonal changes, and no significant differences between means of animals (individual differences), although tendencies for differences between animals of different breed were indicated.

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[4] The advice of Dr. I. M. Lerner, Division of Poultry Husbandry, regarding the statistical treatment of these data is gratefully acknowledged.

Vitamin A levels increased immediately after administration of shark-liver oil to pasture animals (PA group). Animals of the B group continued to show decreasing vitamin A levels until values near zero were found 4 to 5 months after feeding of the carotenoid and vitamin A-deficient diet was begun. Animals receiving shark-liver oil supplements in addition to this diet (BA group) maintained higher blood levels of vitamin A, which dropped to levels observed in B animals immediately after cessation of vitamin A supplements.

Vitamin A levels of the blood were not only dependent on vitamin A intake and carotenoid levels but also on changes in certain physiological factors, like infection, abortion and calving. Such factors produce fluctuations of the vitamin A level of the blood which are independent from the fluctuations of the carotenoid level. Thus, a significant and sharp drop of vitamin A levels started approximately 2 weeks before parturition or abortion, reached its lowest level (minimum level) a few days after parturition or abortion, when it was followed by a sudden rise to the former level. In many animals no vitamin A at all was found in the blood at this minimum shortly after parturition or abortion,[5] particularly in animals of the B and BA groups, less so in animals of the P and PA groups, where the average minimum level reached a point slightly above the zero level.[6] Certain pathological conditions affected the vitamin A level similarly. These pathological conditions were mainly acute infections, like localized abscesses or gangrenous mastitis. In these cases a sharp drop of vitamin A levels was observed, usually before the symptoms manifested themselves, and normal levels were only restored when the infection subsided. The administration of vitamin A in large doses did not prevent these sudden decreases observed in aborting and calving animals of the PA and BA groups, thus ruling out disturbances of vitamin A conversion as the sole underlying cause for the low blood levels.

Figure 1 attempts to present in a composite graph the foregoing discussed changes of vitamin A and carotenoid levels of the blood.

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[5] Zero values for vitamin A may represent a failure in technique rather than complete absence of vitamin A.

[6] An extended investigation of this change at time of calving has been made on lactating cows in order to ascertain whether this change is of general occurrence. It was found that the same drop of the vitamin A level around time of calving took place in lactating animals, too. The results of this additional study will be published elsewhere.

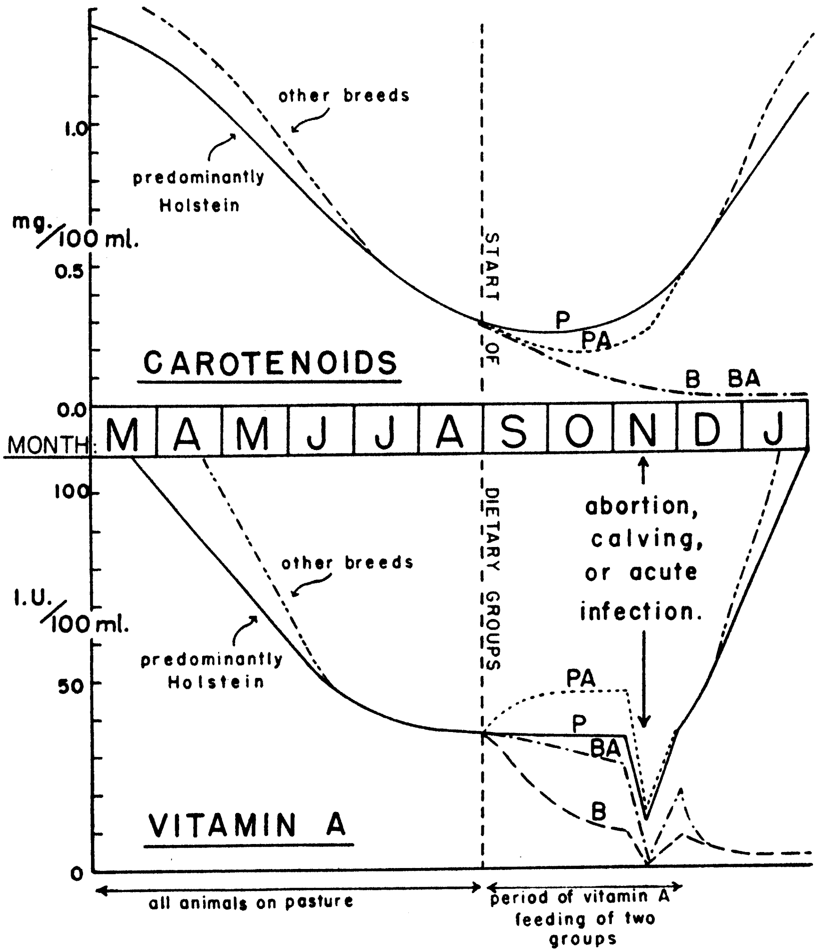

Fig. 1 Diagram showing average changes of carotenoid and vitamin A levels of the bovine blood with changing diet, and during abortion, calving or acute infections. For explanation of dietary groups see text.

The relation of carotenoids to vitamin A in the blood

Since the experiment here reported yielded data on various carotenoid levels with their corresponding vitamin A levels it was possible to analyze the relationship between the two levels under known conditions of carotenoid and vitamin A intake as well as at times of physiological disturbances. Figure 2a illustrates this relationship as found in data obtained from animals which did not receive a vitamin A supplement; therefore, the only source of vitamin A in their blood was the carotenoid intake which had been converted into vitamin A in the liver.

The abcissa of figure 2a represents the carotenoid values; the ordinate, the corresponding vitamin A values. Each point represents the carotenoid and vitamin A value for one test. (Only data obtained from tests made during December to April, when the carotenoid levels generally increased, are used in the graph for reasons to be discussed below.) The distribution of points indicates a linear increase of vitamin A with increasing carotenoid levels (a result, which is not supported by previous work reported by others on cattle). From this graph, a table was constructed which shows the average vitamin A level for each carotenoid level (table 1). By dividing each vitamin A value by its corresponding carotenoid value, a ratio was obtained which indicates in one figure the relative relationship, but should not be interpreted as a direct quantitative measurement between corresponding levels because vitamin A is expressed in I.U./100 ml. and carotenoids in mg./100 ml. here. This will be called the "$\frac{A}{car}$ ratio." Table 1 shows that the $\frac{A}{car}$ ratio decreases with increasing carotenoid levels, reaching a rather constant value around 90 above carotenoid levels of 0.9 mg./100 ml. This decrease of the $\frac{A}{car}$ ratio is graphically indicated in figure 2b. The decrease in the ratio with increasing carotenoid levels may be due to decreasing efficiency of conversion of carotenoids into vitamin A with increasing intake and/or a decreasing release of vitamin A stores from the liver with increasing carotenoid levels.

TABLE 1

Minimum normal $\frac{A}{car}$ ratios.

CAR MG./100 ML.	A I.U./100 ML.	$\frac{A}{CAR}$ RATIO	CAR MG./100 ML.	A I.U./100 ML.	$\frac{A}{CAR}$ RATIO	CAR MG./100 ML.	A I.U./100 ML.	$\frac{A}{CAR}$ RATIO
0.05	25	500	0.50	51	102	0.95	87	92
0.10	26	260	0.55	55	100	1.00	91	91
0.15	27	180	0.60	59	98	1.05	95	90
0.20	29	145	0.65	63	97	1.10	100	90
0.25	31	124	0.70	67	96	1.15	104	90
0.30	35	117	0.75	71	95	1.20	108	90
0.35	39	111	0.80	75	94	1.25	112	90
0.40	43	108	0.85	79	93	1.30	116	89
0.45	47	104	0.90	83	92	1.35	120	89

Deviations from the typical $\frac{A}{car}$ ratios were found under certain conditions. In animals which did not receive vitamin A as such, $\frac{A}{car}$ ratios lower than normal were found during abortions and parturitions, as well as at time of known pathological disturbances. In figure 2a the crosses indicate carotenoid and corresponding vitamin A levels at time of abortion or parturition. These points lie well below the normal distribution, since the vitamin A values are lower than would be expected for each carotenoid level. During the discussion of vitamin A changes at times of abortion and parturition, it has been shown that the vitamin A level dropped considerably. The calculation of the $\frac{A}{car}$ ratio now shows that it is unnecessary to follow this drop in vitamin A by a number of tests in order to show the decrease of the vitamin A level of the blood. One test at the critical time is sufficient to indicate a deviation from the normal $\frac{A}{car}$ ratio and thus establish the presence of physiological disturbances.

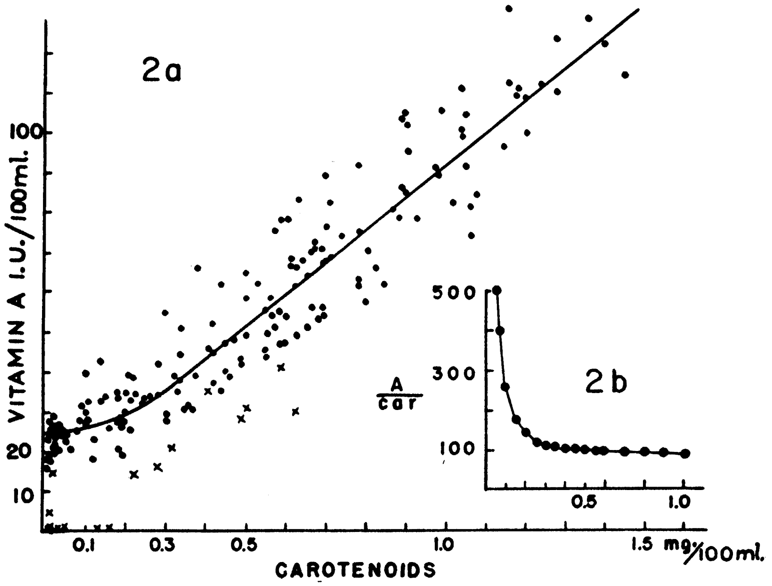

Fig. 2 *a*: Correlation of carotenoids and vitamin A in the blood. Abscissa: carotenoid values; ordinate: corresponding vitamin A values. *b*: Graphical representation of the decrease of the $\frac{A}{car}$ ratio with increasing carotenoid levels. Abscissa: carotenoid values: ordinate: $\frac{A}{car}$ ratios.

$\frac{A}{car}$ ratios above normal were encountered at various times and are best illustrated by a few examples. During a period of steady increase in carotenoids, the $\frac{A}{car}$ ratio follows the normal decrease of values presented in table 2. Upon inspection of a series of values obtained from tests at more frequent intervals, it becomes apparent that the $\frac{A}{car}$ ratio increases above normal for the respective carotenoid level as soon as the carotenoid level drops (see examples in table 2). (This probably means that a decrease of the carotenoid level causes an immediate release of additional vitamin A stores from the liver.) Apparently it does not matter from which level the carotenoid decrease starts. The $\frac{A}{car}$ ratio always increases at such time, regardless of the size of the carotenoid level. This is the reason why it was mentioned above that only data obtained during periods of increasing carotenoid levels were used in establishing the normal $\frac{A}{car}$ ratio. It should be recognized that the initial rise of the $\frac{A}{car}$ ratio at times of carotenoid decrease is due to the fact that the vitamin A level does not exactly correspond to the carotenoid level tested at the same time. In other words, the vitamin A found in the blood originates from carotenoids carried by the blood to the liver some time ago where it has been converted first before appearing as vitamin A in the blood. The vitamin A level, therefore, will show changes which actually correspond to previous carotenoid changes. However, the increase of the $\frac{A}{car}$ ratio at times of decreasing carotenoid levels, as illustrated in table 2, persists for longer periods than could be

TABLE 2

Examples of the effect of increasing and decreasing carotenoid levels upon the $\frac{A}{car}$ ratio.

COW NO.	DATE OF TEST	CAR MG./ 100 ML.	A I.U./ 100 ML.	$\frac{A}{CAR}$ RATIO	COW NO.	DATE OF TEST	CAR MG./ 100 ML.	A I.U./ 100 ML.	$\frac{A}{CAR}$ RATIO
Increasing carotenoid levels									
313	12/14/42	0.14	42.5	304	273	12/14/42	0.19	27.7	146
	1/ 4/43	0.50	48.7	97		1/ 5/43	0.28	34.4	123
321	11/10/42	0.28	41.7	149	279	12/ 4/42	0.34	51.5	150
	1/13/43	1.52	145.4	96		3/19/43	0.89	85.8	96
Decreasing carotenoid levels									
4	2/ 9/43	0.90	95.3	106	308	2/19/43	0.97	91.4	94
	2/16/43	0.99	105.4	106		2/26/43	1.04	100.1	96
	3/10/43	0.58	105.9	183		3/ 9/43	0.44	64.0	145
302	1/29/43	0.26	33.7	130	316	1/21/43	1.24	111.8	90
	2/ 3/43	0.24	33.1	138		2/ 5/43	1.18	108.8	92
	2/ 9/43	0.34	43.9	129		2/12/43	1.14	125.4	110
	2/16/43	0.45	54.8	122		2/17/43	1.16	137.9	119
	3/ 8/43	0.22	41.1	187		3/ 9/43	0.42	52.2	124
292	2/ 3/43	0.44	62.1	141	312	2/ 9/43	0.78	80.7	103
	2/18/43	0.63	83.3	132		2/18/43	1.16	111.5	96
	2/26/43	0.78	74.7	96		2/26/43	1.28	123.4	96
	3/ 8/43	0.45	62.6	139		3/10/43	0.70	125.7	180
	4/ 6/43	0.71	75.0	106		4/ 7/43	1.20	110.1	92

attributed to the difference in time of corresponding carotenoid and vitamin A levels and some other mechanism, such as the suggested additional output of liver vitamin A reserves, must be held responsible. This is further substantiated by a subsequent test which revealed that the principle of increased ratios at time of decreasing carotenoid levels apparently does not apply to animals with fairly depleted vitamin A stores. Four of the animals which had been maintained on the carotenoid and vitamin A-free diet for over 8 months were periodically released on pasture and after a few days returned to the deficient diet. Semiweekly tests on these vitamin A depleted animals subject to fluctuating carotenoid intake revealed no increase in the $\frac{\text{A}}{\text{car}}$ ratio when the carotenoid levels decreased.

Finally, considerably higher $\frac{\text{A}}{\text{car}}$ ratios than those compiled in table 1 were always found in animals which received a shark-liver oil supplement. Obviously, these higher ratios are caused by the addition of ingested vitamin A to the normally present vitamin in the blood, since it is known that vitamin A taken into the body with the feed will be absorbed by the blood from the intestines, part of it transported into the liver for storage and a part utilized immediately by the animal (With, '40).[7] Aside from fed vitamin A, at least one other nutritional factor appeared to be able to elevate the vitamin A level of the blood, thereby causing a considerably higher $\frac{\text{A}}{\text{car}}$ ratio than normal, namely feeding of yeast; it caused an immediate 250 to 300% increase of the vitamin A level after daily feeding of 250 gm. to two animals which had been maintained in the B group for 7 months. Abels, Gorham, Pack and Rhoads ('41) were the first to report this effect in man and suggested that it may be caused by a mobilization of vitamin A stores from the liver due to lipotropic substances contained in the yeast.

It is well known that carotenoid and vitamin A levels differ among breeds (Boyer et al., '42), particularly in the milk, and differences in seasonal changes of the blood level caused by the breed of the animal have been mentioned above. Therefore, the question arises whether these breed differences may not affect the $\frac{\text{A}}{\text{car}}$ ratio. However, the animals studied in this experiment do not represent a genetically homogenous group; still, the relative $\frac{\text{A}}{\text{car}}$ ratio did not show any differences for animals of known different ancestry. Furthermore, samples obtained from Hereford and Jersey cattle at the slaughterhouse revealed similar $\frac{\text{A}}{\text{car}}$

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[7] This point is of importance for tests in which the vitamin A status of an animal is being tested by blood-tests. Care has to be taken at the time of such a test to see that no vitamin A is being taken in by the animal since such an intake would mask the actual release of stored or converted vitamin A from the liver.

ratios for corresponding carotenoid levels. Thus it seems that breed differences are expressed in different seasonal levels which apparently do not affect the relative ratio of vitamin A to carotenoids.

SUMMARY

The carotenoid and vitamin A levels of the blood of forty-nine cows maintained in four dietary groups with different carotenoid and vitamin A intake, were determined periodically for 1 year.

The seasonal changes of the carotenoid level were mainly dependent on the diet, i.e., carotenoid intake, but varied according to the age and breed of the animal. Individual difference, i.e., differences between the means of animals, were found to be statistically significant. Pathological disturbances showed no direct effect on the carotenoid level.

The seasonal changes of the vitamin A level were dependent on carotenoid and vitamin A intake and were modified during parturition, abortion and acute infections at which time vitamin A levels showed a sharp, temporary decrease. No statistically significant difference was found between individual animals or age groups, but a difference between breeds was indicated. The vitamin A level rose immediately after administration of shark-liver oil and, according to a preliminary test, after feeding of yeast.

The relation of carotenoids to vitamin A in the blood was analyzed. A linear increase of vitamin A with increasing carotenoid levels was found. The ratio of vitamin A to carotenoids at various carotenoid levels was established. This $\frac{A}{car}$ ratio was found to decrease with increasing carotenoid levels, reaching a constant value at high carotenoid levels. Deviations from the normal $\frac{A}{car}$ ratios were found and analyzed. The $\frac{A}{car}$ ratio was below normal in cases of physiological disturbances (parturition, abortion, acute infections), slightly above normal if animals were subject to decreasing carotenoid intake, and well above normal if a vitamin A supplement was fed. The effects of breed differences on the $\frac{A}{car}$ ratio are briefly discussed.

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STUDIES ON THE CAROTENOID AND VITAMIN A LEVELS IN CATTLE

II. CAROTENOIDS AND VITAMIN A IN THE LIVER, THEIR RATIO AND THEIR RELATIONSHIP TO BLOOD LEVELS

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TWO FIGURES

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INTRODUCTION

Seasonal changes of the carotenoid and vitamin A levels of the blood of a group of cattle maintained on various dietary regimes were reported in a previous paper (Braun, '45). Data were also obtained on the carotenoid and vitamin A content of the livers of animals used in this study. These data will be discussed in this report, together with additional data obtained from slaughterhouse material which was collected in order to establish the relationship between vitamin A levels of the liver and the blood.

The vitamin A and carotenoids in liver tissue were determined by Guilbert and Hart's ('34) method which was modified for use with the Cenco photometer.

Liver samples were obtained at time of slaughter or, in a few cases, by partial hepatectomy. Blood samples were always collected at the same time and their carotenoid and vitamin A content was determined according to methods described in an earlier report (Braun, '45).

The data obtained from the University of California cows gave information about the effect of vitamin A feeding on the liver storage of vitamin A, because the animals were maintained in four dietary groups: (1) a pasture group (P group); (2) a pasture group in which each animal received 400,000 I.U. of vitamin A, in the form of shark-liver oil, twice a week from September through November (PA group); (3) a barn group, transferred on September 1st from pasture into the barn

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TABLE 1

Carotenoids and vitamin A in the liver and the corresponding blood levels.

DIETARY GROUP (SEPT.–NOV.)	COW NO.	DATE OF TEST	CAR. µG./GM.	A I.U./GM.	CAR. MG./100 ML.	A I.U./100 ML.
A. Data obtained during and shortly after period of shark-liver oil feeding.						
P	263B	11/ 5/42	13.4	244.7	. . .	. . .
	3	11/25/42	6.2	195.8	0.32	21.2[1]
		2/ 1/43	10.4	262.8	0.61	39.5
	315	12/17/42	7.6	256.8	0.44	45.0
	305	1/ 5/43	8.4	279.2	0.42	51.8
	341	1/ 5/43	10.0	256.3	0.50	65.0
	313	1/ 5/43	7.2	253.2	0.50	48.7
	2	12/17/42	4.8	220.6	0.44	47.9
				M = 246.2		
B	319	12/ 2/42	2.4	120.8	0.00	0.0[2]
	343	12/15/42	1.4	94.0	0.01	10.5[3]
	200	1/11/43	1.4	96.8	0.03	19.8
	(284	1/19/43	1.6	181.3	0.02	0.0)[4]
				M (without 284) = 103.9		
PA	273	1/ 5/43	8.4	449.4	0.28	34.4
	326	1/ 5/43	16.0	342.9	0.90	101.6
				M = 396.2		
BA	282	12/ 1/42	7.4	335.8	0.01	0.0[5]
		2/ 1/43	4.0	341.1	0.01	24.6
	293	12/15/42	2.6	265.8	0.05	0.0[6]
	318	1/ 5/43	5.2	396.5	0.30	34.8
				M = 334.8		
B. Data obtained 6 to 8 months after cessation of shark-liver oil feeding.						
P	309	5/25/43	16.0	409.5	0.59	61.3
	314	7/15/43	8.0	385.3	0.26	51.1
	342	7/15/43	7.0	333.3	. . .	. . .
				M = 376.0		
B	312	5/25/43	20.8	339.4	0.78	99.0
	343	5/25/43	6.4	118.7	0.42	63.6[7]
	247	7/15/43	4.2	297.4	0.23	33.8
	327	7/15/43	6.2	142.4	0.31	36.4
				M = 224.5		
PA	4	5/25/43	20.4	522.9	0.82	87.5
	207	7/15/43	14.8	578.4	0.45	50.4
				M = 550.3		
BA	(292	5/25/43	9.6	293.1	0.55	60.9)[8]
	1369	7/15/43	8.6	464.5	0.17	27.0

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[1] Liver sample obtained by partial hepatectomy after abortion on 11/24/42.

[2] Liver sample obtained by partial hepatectomy after abortion on 12/1/42.

[3] Liver sample obtained by partial hepatectomy after abortion on 12/14/42.

[4] Died in corral. Autopsy revealed severe peritonitis and adherent pericardium, also inflamed areas in intestine.

[5] Liver sample obtained by partial hepatectomy after abortion on 11/28/42.

[6] Extensive edema of abdomen and brisket, with a huge slough in the right axillary region at time of slaughter.

[7] Released on pasture on 3/10/44.

[8] Pericarditis, foreign body lesions revealed by autopsy on 5/25/43.

where they were maintained on a carotenoid and A-deficient diet (B group; and (4) a group of barn animals which received a shark-liver oil supplement (400,000 I.U. twice a week) from September through November (BA group). A more detailed description of these animals, their diet and the changes observed in their carotenoid and vitamin A levels of the blood have been presented previously (Braun, '45).

RESULTS AND DISCUSSION

The values obtained from liver samples of these animals are presented in table 1, where they are arranged according to dietary groups. Data collected during November through January, i.e., during and immediately after the period of shark-liver oil feeding, are grouped separately from data obtained from animals slaughtered 6 to 8 months later. The November–January data were then used for a calculation of ratios of vitamin A between the averages of the different dietary groups (table 2). Values obtained after partial hepatectomy are incorporated in table 1.

TABLE 2

The ratio of average vitamin A storage between the four dietary groups.

GROUP	RATIO OF AVERAGE VITAMIN A BETWEEN THE GROUPS
Barn fed: Barn fed plus shark-liver oil	1:3.5
Barn fed: Pasture fed	1:2.4
Barn fed: Pasture fed plus shark-liver oil	1:4.1
Pasture fed: Barn fed plus shark-liver oil	1:1.4
Pasture fed plus shark-liver oil: Barn fed plus shark-liver oil	1:0.9
Pasture fed: Pasture fed plus shark-liver oil	1:1.6

An inspection of these data indicates: (1) considerable differences in liver vitamin A levels between animals of the B group, the P group and both BA and PA groups, with less significant differences between the two latter groups (upper part of table 1); (2) a rather lasting effect of the relatively short period of vitamin A feeding on the liver storage, since the group differences were still found in animals which had been on identical diets for at least 4 months before slaughter which was 6 to 7 months after cessation of shark-liver oil feeding (lower part of table 1); and (3) an optimum level for vitamin A storage, suggested by the ratios in table 2, which show that levels of the BA group were 3.5 times higher than those of the B group, while those of the PA group were only 1.6 times higher than those of the P group, both groups having received equal amounts of shark-liver oil. (If there would not be an optimum level for vitamin A storage, the vitamin A storage of the PA group should be considerably higher after vitamin A feeding than that of the BA group, because the pasture animals without vitamin A supplement had 2.4 times as much vitamin A in their livers as the barn animals without vitamin A supplement.) Next, it should be noted that uniformly low carotenoid levels were observed only in animals without carotenoid or vitamin A intake (B group). Results obtained with cow no. 282 are particularly interesting in this connection. The caudate liver lobe of this animal had been removed on December 1st [2] and its carotenoid and vitamin A content had been estimated at 7.4 µg./gm. and 335.8 I.U./gm., respectively. Two months later this animal was slaughtered after having been maintained on the carotenoid and vitamin A-free diet of the barn group. At the time of slaughter, the carotenoid level of the liver was found to be 4.0 µg./gm., indicating an almost 50% decrease of the carotenoid content. However, the vitamin A content had remained unchanged; the value obtained was 341.1 I.U./gm. More experimental work appears necessary to substantiate the suggestion that a utilization of stored vitamin A first forces available carotenoids to be converted into vitamin A, thus first decreasing the carotenoid level without decreasing the vitamin A level, and only after a very low carotenoid level has been reached will a drain on vitamin A stores become apparent by decreased vitamin A storage. The increase of liver stores when green feed becomes available on pasture after January is illustrated in the results of partial hepatectomies performed on cows no. 3 and no. 343 (table 1).

Similarly, to the $\frac{A}{car}$ ratios observed in the blood (Braun, '45), a typical relationship between carotenoid levels and corresponding vitamin A levels appears to exist in the liver. The ratio of each vitamin A level to its corresponding carotenoid level was calculated, and the resulting ratios were plotted against increasing carotenoid levels (fig. 1). The distribution of the dots in this graph, representing values obtained from animals without vitamin A supplement, indicate a decrease in the $\frac{A}{car}$ ratio of the liver with increasing carotenoid levels. Each carotenoid level has its typical $\frac{A}{car}$ ratio. The above cited decrease of the carotenoid level without changes in the vitamin A level, i.e., the metabolic tendency of the organism to maintain a constant vitamin A store, probably causes the rapid rise in $\frac{A}{car}$ ratios with decreasing carotenoid levels. The $\frac{A}{car}$ ratios of the liver were modified if the animals were fed vitamin A, as was the case in the animals of the barn and pasture groups which had been fed a shark-liver oil supplement. The circles on the graph represent the values obtained for the $\frac{A}{car}$ ratio of these shark-liver oil-fed animals. The $\frac{A}{car}$ ratio of all these animals is constantly larger for each carotenoid level compared with ratios of animals which did not get the supplement. This can be easily explained: the vitamin A fed has been added to the normally present vitamin A stores, thus modifying the $\frac{A}{car}$ ratio. This modification of the normal $\frac{A}{car}$ ratio of the liver under conditions where vitamin A is supplied with the feed explains why investigators like Ralli et al. ('41) were unable to detect constant $\frac{A}{car}$ ratios in their human liver material. The human diet contains varying

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[2] This operation was performed by Dr. B. N. Carle.

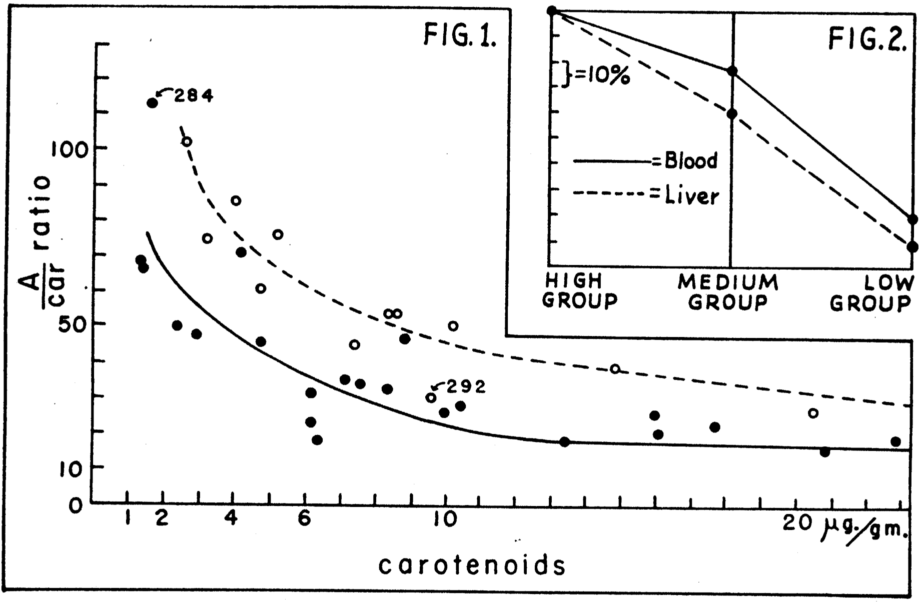

Fig. 1 Graphical representation of the decrease of the $\frac{A}{car}$ ratio of the liver with increasing carotenoid levels. Dots represent data from animals maintained without vitamin A supplement; circles represent data from animals which received vitamin A supplement. Abscissa: carotenoid values; ordinate: $\frac{A}{car}$ ratios.

Fig. 2 Graphical representation of the relationship between vitamin A levels of the liver and those of the blood, using the means of data presented in table 3. Abscissa: dietary groups; ordinate: vitamin A levels in per cent of decrease.

amounts of vitamin A in addition to carotenoids and this fluctuating intake of vitamin A modifies the normal $\frac{A}{car}$ ratio of the liver as well as that of the blood (Braun, '45). The above mentioned observation that temporary feeding of vitamin A had a rather lasting effect on the increased vitamin A storage makes it possible to detect administration of vitamin A supplements, through liver tests and comparison with normal $\frac{A}{car}$ ratios, made an appreciable period after the supplement has been stopped. A number of workers have shown that the liver vitamin

A is decreased under various pathological conditions (Barron, '42; Ralli and co-workers, '41; With, '40). The liver material here investigated contained three specimens obtained from animals at the time of pathological disturbances: namely cows no. 284, no. 292 and no. 293 (table 1). Two of them (no. 284 and no. 292) showed unusual $\frac{\text{A}}{\text{car}}$ ratios of the liver. Blood samples from cows no. 184 and no. 293 showed an abnormal $\frac{\text{A}}{\text{car}}$ ratio at the time when the liver samples were obtained. Liver specimens from animals no. 3, no. 282, no. 319 and no. 343 were obtained immediately after abortion. No abnormal $\frac{\text{A}}{\text{car}}$ ratio was found in these livers at that time, while the $\frac{\text{A}}{\text{car}}$ ratio of the blood was abnormal at this period (Braun, '45).

TABLE 3

Mean vitamin A levels of the liver and of the blood for three groups of animals maintained on diets with different vitamin A intake.

GROUP	NUMBER OF ANIMALS	MEAN OF VIT. A IN LIVER	MEAN OF VIT. A IN BLOOD
High group	37	69.2 ∓ 5.53	20.4 ∓ 1.18
Medium group	6	42.8 ∓ 4.09	16.1 ∓ 1.65
Low group	9	19.5 ∓ 7.35	6.7 ∓ 2.68

No correlation exists normally between the carotenoid and vitamin A values of the liver and those of the blood. Blood tests were made on the day of slaughter, and the resulting carotenoid and vitamin A data compared with the results of the liver tests (table 1). The only time that low vitamin A stores were reflected by low vitamin A levels in the blood was during periods of rapid depletion of vitamin A stores of the liver without simultaneous intake of carotenoids or vitamin A, as was the case in the barn group without shark-liver oil supplement.

This limited observation was further substantiated by material obtained from the slaughterhouse. Livers and blood samples were collected from fifty-two animals, which were used in an experiment in which the effect of different vitamin A diets on the incidence of liver abcesses was studied.[3] The animals (Hereford cattle) had been kept in feed lots in three groups: high vitamin A diet, medium vitamin A diet, and low vitamin A diet. The means for the vitamin A stores found in each of the three groups and the means for the vitamin A levels of the blood of each group are presented in table 3, and the means plotted, in terms of percentage of decrease in vitamin A levels between groups, are shown in figure 2. It can be seen that a tendency towards a direct relationship between vitamin A stores and the vitamin A level of the blood exists only when the former falls below normal levels.

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[3] This study was conducted by Drs. H. S. Cameron and G. H. Hart. Acknowledgment is made for their kind cooperation in collecting these samples.

SUMMARY

Carotenoid and vitamin A values were obtained from liver samples of cows maintained in four dietary groups with different carotenoid and vitamin A intake. The vitamin A levels of the liver were significantly different among three of the four dietary groups, but similar for the shark-liver oil supplemented groups regardless of their basic diet. An optimum level for vitamin A storage was thus indicated.

Results obtained from livers of animals which had been maintained on a vitamin A enriched diet 6 to 8 months previously indicated a rather lasting effect on the vitamin A storage of a relatively short period of vitamin A feeding.

According to observations made on livers from vitamin A-starved animals and on samples obtained by partial hepatectomy, utilization of stored vitamin A first forces available carotenoid stores to be converted into vitamin A, thus decreasing the carotenoid level without decreasing the vitamin A level.

Similarly to the $\frac{A}{car}$ ratios observed in the blood, a typical relationship between carotenoid levels and corresponding vitamin A levels appears to exist in the liver. Changes in the ratio with changing carotenoid levels are probably caused by the tendency of the organism to maintain a constant vitamin A store. The $\frac{A}{car}$ ratio of the liver was found to be modified if the animal was fed vitamin A and in certain pathological conditions.

A tendency towards a direct relationship between vitamin A stores and the vitamin A level of the blood was found to exist only when the former fall below normal levels.

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EDITORIAL REVIEW

THE REFINEMENT OF METABOLIC CALCULATIONS FOR NUTRITIONAL PURPOSES AND THE PROBLEM OF "AVAILABILITY"

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The supply and distribution of food under government control in war time makes great demands on nutritional science. The calculation of caloric requirements and the efficiency of food utilization assumes a new importance when the results form the basis of international food shipments and ration allotments. Even a very small percentage of millions of tons represents an impressive amount of food so there is an understandable desire to refine caloric calculations to the utmost. Such refinement involves several considerations.

Obviously the first step is to obtain more accurate analytical data on the composition of all foods which contribute significantly to the total diet. This task has been undertaken by groups of experts in different countries, notably England, Canada and the United States. Improved tables on food composition are now being made available in this country by the National Research Council. In general, such tables provide information on the food content in water, ash, fats and nitrogen as estimated by chemical procedures. The carbohydrate is obtained by difference, frequently with some correction for "fiber" or "digestibility."

Tables of food composition are used for the estimation of dietary adequacy and of human requirements in terms of specific foods. Our real concern is with the physiological results obtained from carbohydrates, fats and proteins and these may not be exactly indicated by the amounts of these substances measured by the usual analytical methods. McCance and Widdowson ('42) advocate corrections for "availability"of the carbohydrates and their ideas have influenced the views of the British experts so that American and British calculations for the caloric value of the current U.S.A. diet systematically differ by something like 5%.

Dr. L. A. Maynard ('44) has discussed the basis of classical caloric calculations and has shown that McCance and Widdowson overlook the fact that the standard calorie: gram factor for carbohydrates — 4.0 : 1 — already contains a reasonable over-all correction for "availability" of carbohydrate calories in the mixed diet. It will be agreed that in nutritional calculations we should like to use values which would more precisely allow for true availability, but the McCance and Widdowson figures appear to increase the errors for the total diet though they may improve the figures for some individual foodstuffs. However, even if the McCance and Widdowson tables were more properly constructed their use would not eliminate several sources of uncertainty.

The true problem of availability is not limited to carbohydrates nor to matters of simple digestibility. The physiological utilization of food is rarely important solely in terms of total calories or heat units. The uses of food for the purposes of growth, tissue maintenance and physical work are generally more important and these functions are not quantitatively related by an unvarying factor to the total heat. There are good reasons to believe that the efficiency of endogenous utilization of different nutrients is altered by the physiological destination of the energy involved.

There is no intention to question the equivalence of direct and of indirect calorimetry but the forms and places of energy conversions are of consequence. Neglect of these questions may lead to errors which, though relatively small, seem more significant as nutritional calculations become more exact. Unfortunately acceptable data in this field are few but several simple examples may be cited.

Both corn starch and glucose are completely "digestible" but their total heats of combustion per unit weight differ by 15%. For 100 gm. of these substances the total heats of combustion are 429.71 and 373.69 Cal., respectively. The first stage of the metabolism of starch is its hydrolysis, and part of the difference in total heat of combustion between starch and glucose is represented in heat of hydrolysis amounting to about 14.48 Cal. for 100 gm. of corn starch. If some of this hydrolysis takes place in cooking the food the corresponding heat of hydrolysis is completely lost. Ordinarily most of the hydrolysis takes place in the gastro-intestinal tract and this provides body heat which would appear in any total calorimetric measurement. But this energy is not "available" for any other purpose. It is clear that no single factor for the calculation of carbohydrate calories may be applied to both starch and glucose without significant error, and even if two different factors are used it is necessary to specify the destination of the calories for rigid accuracy.

It is known that appreciable amounts of alcohol are readily metabolized to yield heat but it has been argued that calories of such dubious antecedents cannot be utilized to support growth or muscular activity. It now appears that alcohol at least may spare other nutrients so they may participate in growth (Mitchell, '35) and that alcohol may even provide energy for muscular contraction (Sommerkamp, '24; Grubbs and Hitchcock, '38). The efficiency in either process is unknown. The question is not entirely trivial when we note that alcohol may provide as much as 10 or even 20% of the total calories of the diet for some groups in northern and central Europe.

A more important example is the question of the efficiency of fats as fuel for muscular work. In this laboratory we have been able to confirm the findings of Krogh and Lindhard ('20) and of Bierring ('32) that, calorie for calorie, fat is less efficient than carbohydrate in the production of external work. The over-all difference is about 12%, that is about 112 cal. of mixed fats must be used to produce the same number of kilogram meters of external work as produced by 100 cal. of mixed carbohydrates. Since under the most favorable conditions muscular work is only about 25% efficient, it appears that the actual metabolic pathway — fat calories to muscular contraction calories — involves an excess wastage of about 16%. On the other hand, there is no difference if the calories are needed solely to maintain body temperature. The significance of these facts appears if we consider that on the average from 40 to 50% of all food calories are used in the production of muscular work and that in heavy industry and in military operations as much as 75% of the total calories may go into work. In contrast as little as 10% of the energy expenditure of the hospital patient may be devoted to muscular activity.

The few exact studies which have been made on the fuel of muscular work in man do not differentiate sufficiently between different types of fats and different types of carbohydrates. There are marked differences in rates of metabolism of different sugars and quite likely somewhat different metabolic pathways are involved. It is probable that these differences are reflected in differences in physiological economy. Nor do we have adequate information as to the relative efficiency of proteins in the support of muscular work. In terms of total efficiency what is to be done with the calories of specific dynamic action?

Under some ideal laboratory conditions the calculation of caloric requirements may be made with considerable accuracy but in real life situations the errors are large. Even painstaking calculations with small homogeneous groups frequently lead to apparent absurdities. The recent study by Wiehl ('44) provides examples. Comparison of food intake versus elaborately computed "requirements" indicated that 55.3% of 272 private school pupils were undernourished but of these supposedly calorically deficient pupils only 11.1% were underweight while 47.7% of them were overweight by 7.5% or more. The incomplete data on public high school pupils suggest even greater discrepancies.

In this brief discussion it has not seemed necessary to consider the natural variability of foods which imposes an inherent limitation to the detailed accuracy of any tables of composition when applied to any particular sample or diet. The study of nutrition has made enormous strides in recent years but it is well to reflect on the limitations of present knowledge in making quantitative applications to man. There is some danger that revision of caloric tables to include "available carbohydrate" in the old sense may lead to a false sense of accuracy. Much more research, especially quantitative studies on man, is needed to create a true science of nutrition.

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EFFECTS OF LIGHT INTENSITY, DAY LENGTH, TEMPERATURE, AND OTHER ENVIRONMENTAL FACTORS ON THE ASCORBIC ACID CONTENT OF TOMATOES

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Variations in the ascorbic acid content of ripe tomatoes are known to be large (Hamner and Maynard, '42). The review of the literature revealed, however, that the data were highly conflicting as to the factors responsible for the variations. It was evident that the majority of the studies had not been sufficiently well controlled to determine the effects of specific factors and their possible interrelations. Therefore, a study was begun in this laboratory 4 years ago in the hope of obtaining specific information which could be utilized in practice to provide consumers with tomatoes of a higher and more reliable nutritional quality. This study has concentrated on environmental factors. Most of the work has been done with an inbred strain of Bonny Best tomatoes and has stressed the use of carefully established conditions so that as many environmental factors as possible were under control.

A study of the variations in carotene content of tomatoes has appeared in a separate report (Ellis and Hamner, '43). In the present report, results are presented of a series of experiments, some of which involve field grown plants and others which were conducted under controlled conditions. The work demonstrates that very large variations in ascorbic acid content of tomatoes may be associated with growing conditions and indicates that a factor of primary importance in determining the ascorbic acid level may be the light intensity a few days previous to harvest.

Unless otherwise stated, the fruits were analyzed on the day that they became red over the entire surface. The methods used in sampling and analyzing were those previously described (Hamner et al., '42). Many individual fruits were analyzed in obtaining each mean value, and the number of fruits, together with the standard error, is presented. All

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values are presented in milligrams of ascorbic acid per 100 gm. of fresh fruit.

Variations in fruit from different locations

In previous studies (Hamner et al., '42), it was found that plants of an inbred strain of Bonny Best tomatoes, grown at five widely separated locations in the United States, produced fruit which differed markedly in ascorbic acid content. These variations in ascorbic acid content, associated with location, could not be correlated with soil conditions or cultural practices, and presumably were related to climatic differences from one location to another.

Because of the results just mentioned, fruits from many locations were analyzed in order to determine the magnitude of the variations in ascorbic acid which might be associated with climatic conditions during the growing season. Since all our experiments with tomatoes have indicated that soil variables produce relatively little effect upon ascorbic acid, fruit from plants grown in soil under the cultural practices prevalent at a given location was obtained for analyses. The fruits were picked when ripe, carefully packed in paper, and shipped by express to Ithaca, New York, for analysis.[1]

The fruits were analyzed as soon as they arrived at Ithaca. While some lots of fruit were doubtless picked at more advanced stages of ripeness than others, and some were a longer time en route than others, these factors were probably not of great importance in determining the differences in ascorbic acid values obtained. Work of others (as reviewed by Hamner and Maynard, '42), as well as work described later in this report, indicates that the changes in ascorbic acid content during storage for a week at room temperature and during the later stages of ripening are not great unless the fruits are obviously "over-ripe". All of these samples were firm and at the "red-ripe" stage.

The result of the analyses are given in table 1. Variations in ascorbic acid content are large even for the same variety. Fruits of Marglobe varied from 14.4 ± 2.12 to 30.6 ± 2.04; of Rutgers, from 8.4 ± 0.50 to 19.7 ± 1.17; of Pritchard, from 10.7 ± 0.88 to 29.0 ± 1.29. Of the two varieties, Marglobe and Rutgers, the former consistently had more ascorbic acid at any particular location, except at Madison, Wisconsin. Both Pritchard and Marglobe were available at only three locations. At two, Pritchard had about 30% less ascorbic acid, and at one the values were about the same. At two locations, Pritchard and Rutgers had about the same ascorbic acid values.

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[1] Grateful acknowledgment is made to numerous individuals who cooperated in supplying these fruits.

The results of the analyses are given in table 1. Variations in ascorbic 30.6 ± 2.04. While the nature of the experiment is such that the exact cause of these variations cannot be ascertained, it seems probable that environmental differences among locations were more important than varietal differences.

TABLE 1

Ascorbic acid content of tomatoes raised in various parts of the United States during the summer of 1942.[1]

LOCATION	DATE ANALYZED	VARIETY					
		Marglobe		Rutgers		Pritchard	
		Mean ± S.E.	No. of analyses	Mean ± S.E.	No. of analyses	Mean ± S.E.	No. of analyses
Madison, Wis.	9/11	17.2 ± 0.76	14	19.0 ± 1.09	13	18.9 ± 1.52	14
Kingston, R. I.	8/29	15.5 ± 0.75	15	8.4 ± 0.50	6	10.7 ± 0.88	11
Holgate, Ohio	9/1	22.3 ± 0.90	13	19.4 ± 0.90	12	....	..
East Lansing, Mich.	9/1	16.7 ± 0.67	15	14.2 ± 0.55	15	....	..
State College, Pa.	8/31	15.7 ± 0.63	15	14.5 ± 0.70	13	....	..
Ames, Iowa	9/4	18.4 ± 1.20	14	15.6 ± 0.60	13	....	..
St. Paul, Minn.	9/8	19.5 ± 1.30	10	....	..	14.1 ± 0.78	14
Fresno, Calif.	9/8	....	..	....	..	29.0 ± 1.29 [2]	10
Orono, Maine	9/21	....	..	....	..	19.8 ± 1.13	16
Knoxville, Tenn.	8/29	14.4 ± 2.12	3	....	..	....	..
Wenatchee, Wash.	9/1	30.6 ± 2.04	12	....	..	....	..
Auburn, Alabama	9/2	....	..	18.8 ± 1.50	9	....	..
Charleston, S. C.	9/11	18.9 ± 1.12	10	....	..	....	..
Lafayette, Ind.	9/4	....	..	19.7 ± 1.17	11	....	..

[1] The following results were obtained with various other varieties at different locations:

Location	*Variety*	*Date*	*No. analyzed*	*Mean ± S.E.*
Riverside, Calif.	Stone	9/1	14	20.9 ± 0.81
Riverside, Calif.	Pearson	9/1	15	30.2 ± 0.82
Mt. Carmel, Conn.	John Baer	8/29	16	13.5 ± 0.60
Davis, Calif.	Pearson	8/25	20	16.0 ± 0.67
Davis, Calif.	Santa Clara Canner	8/25	19	22.1 ± 1.13
Lafayette, Ind.	Ind. Baltimore	9/4	5	19.7 ± 1.61

[2] Twelve fruits were picked when partially ripe and gave values averaging 26.8 ± 1.27.

Degree of ripeness and length of storage

The Pritchard variety, grown in experimental gardens of the Department of Vegetable Crops, Cornell University, were used in these experiments.[2] All fruits were picked September 2, 1940. Fruits at various stages of ripeness were picked as follows: 8 red, soft and obviously "over-ripe"; 20 sound, firm, "red-ripe"; 20 partially red; 400

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[2] Acknowledgment is made of the courtesy of Dr. H. C. Thompson for supplying these fruits.

"mature green" (large size, light green with some areas of the surface almost white); 20 "immature green" (about half the size of average ripe fruit). All fruits were analyzed immediately except for 380 of the "mature green" fruits which were used in the storage experiment. These were divided into five lots. A lot of 50 was placed at each of the following storage temperatures: 65°, 70°, and 90°F. An additional lot was placed in storage at 75°F. At each of the storage temperatures, the fruits were analyzed as soon as they turned red over the entire surface. At the higher temperatures some did not turn red, but rather changed from a green to a yellowish pink. These were analyzed when they had lost most of their green color. After 10 days of storage, ten fruits stored at 75°F. were analyzed which were at the "pink" stage, and an additional ten which were still green (table 2).

TABLE 2

Ascorbic acid content of fruit at different stages of ripeness and storage at different temperatures.

TREATMENT	STAGE OF RIPENESS	NO. OF ANALYSES	ASCORBIC ACID MG./100 GM. FRESH WEIGHT	TREATMENT	STAGE OF RIPENESS	NO. OF ANALYSES	ASCORBIC ACID MG./100 GM. FRESH WEIGHT
Initial harvest	Immature green	20	14.0 ± 0.51	*Stored at:*			
	Mature green	20	15.0 ± 0.69	75°—1 week	Red-ripe	11	17.3 ± 1.11
	Pink	20	15.4 ± 0.45	2 weeks	Red-ripe	52	12.9 ± 0.36
	Red-ripe	20	16.2 ± 0.64	3 weeks	Red-ripe	23	7.6 ± 0.38
	Over-ripe	8	16.6 ± 0.91	80°—1 week	Red-ripe	7	14.0 ± 1.58
Stored at:				2 weeks	Red-ripe	25	9.8 ± 0.99
65°—1 week	Red-ripe	4	14.5 ± 2.11	3 weeks	Red-ripe	13	7.1 ± 0.48
2 weeks	Red-ripe	12	15.0 ± 1.78	90°—1 week	Ripe[1]	5	14.0 ± 0.48
3 weeks	Red-ripe	7	8.5 ± 0.39	2 weeks		19	10.8 ± 0.68
70°—1 week	Red-ripe	6	14.4 ± 1.35	75° and removed after 10 days storage	Mature green	10	8.8 ± 0.47
2 weeks	Red-ripe	18	12.9 ± 0.66		Pink	8	13.1 ± 1.30
3 weeks	Red-ripe	10	8.2 ± 0.75		Red-ripe	17	14.5 ± 0.50

[1] Did not beome red-ripe. Analyzed when yellowish pink.

At the time of the initial harvest, the green fruits were only slightly lower in ascorbic acid than the ripe ones. Those which ripened during the first week of storage were approximately as rich in the vitamin as those which were ripe at the initial harvest. Fruits ripening during the second week of storage tended to be a little lower in ascorbic acid, especially at the higher storage temperatures, and those ripening subsequent to 2 weeks of storage, regardless of storage temperature, were appreciably lower in ascorbic acid, and the variability between fruits was much less. After 10 days of storage at 75°F., the degree of ripeness was correlated with marked differences in ascorbic acid, green fruit having values appreciably lower than ripe fruit. Perhaps those individual green fruits which were low in ascorbic acid at the time of the initial harvest were the same ones which ripened last in storage and thus account for some of the apparent decrease during storage. It is also possible that ascorbic acid was destroyed more rapidly during storage in those which were the "greenest" upon entering storage.

Variety and fertilizer treatment

In previous reports (Hamner et al., '42; Lyon et al., '43, '44) the influence of mineral nutrition on ascorbic acid content was found to be of relatively minor importance. While certain levels of supply of some of the elements did affect the ascorbic acid content, the concomitant effects on growth and fruitfulness were so great that it is doubtful if similar levels of supply would occur under field conditions of commercial production.

During the summer of 1942, analyses were made of three varieties grown at the New York State Experiment Station, Geneva, New York. The details of the experimental design, fertilizer treatments, growth and yield data, etc., will be reported by Dr. C. B. Sayre in a separate publication. There were seven fertilizer treatments with three replications of each treatment for each variety, and seven fruits were analyzed from each replicate. The mean values for ascorbic acid content of each treatment are given in table 3. The significant variation seems to

TABLE 3

Ascorbic acid content of tomatoes produced with plants grown at Geneva, New York, on soil receiving seven different fertilizer treatments.

FERTILIZER APPLICATIONS[1]		VARIETY (ASCORBIC ACID IN MG./100 GM. FRESH FRUIT)			
Kind	lbs./acre	Stokesdale	Rutgers	N. Y. State	Treatment Mean
None	None	22.2 ± 0.99	20.8 ± 0.65	20.7 ± 0.68	21.2
0-20-10	600	24.5 ± 0.78	20.9 ± 0.67	23.5 ± 1.10	23.0
5-20-10	600	22.3 ± 1.33	23.0 ± 0.85	21.6 ± 0.58	22.3
20-20-10	600	18.7 ± 0.67	18.0 ± 0.46	19.6 ± 1.55	18.8
5-20-10[2]	600	21.6 ± 0.86	21.2 ± 0.70	21.0 ± 0.84	21.3
5-20-20	600	19.9 ± 0.70	19.9 ± 0.65	20.3 ± 0.57	20.0
3-12- 6[3]	1000	21.2 ± 0.84	20.5 ± 0.46	21.8 ± 0.85	21.2
Varietal mean		21.5	20.6	21.2	

[1] Figures show percentages of available N, P, and K as total N, P_2O_5, and K_2O obtained by analysis of fertilizer.

[2] Sixty pounds of MgO as $MgSO_4$ were also added in this treatment.

[3] Premium grade.

be associated with a heavy application of nitrogen (20-20-10) which resulted in low ascorbic acid values. The differences between this treatment and the others were not great and were probably of little applied significance. In light of the results of an experiment described subsequently, it seems possible that these low values may have been associated with the influence of the heavy nitrogen application in producing greater foliage and resultant shading of the fruit.

Temperature and humidity

In this experiment, use was made of three control chambers previously described (Hamner, '44). The respective temperature and humidity of the chambers was: chamber 1, 78° ± 2°F. and 90 ± 5%; chamber 2, 63° ± 2°F. and 84 ± 5%; chamber 3, 78° ± 2°F. and 30 to 50% relative humidity. Thus, chambers 1 and 2 were operated at two different temperatures with approximately the same vapor pressure deficit (equivalent to 2.3 mm. of mercury). Chambers 1 and 3 were operated at the same temperature with a relatively high and a relatively low humidity, respectively. The chambers were illuminated for 16 hours each day with "white" and "daylight" fluorescent lamps being used alternately (Hamner, '44), the illumination intensities as measured by a Weston foot-candle meter being from 800 to 900 foot-candles. The various environmental factors, other than those under study, were controlled as nearly alike as possible.

Seedlings of the inbred strain of Bonny Best tomatoes were germinated in sand in the greenhouse and the seedlings transplanted singly into two-gallon crocks containing quartz sand. Eight plants were placed in each chamber, and eight more plants were continued on experiment in the greenhouse. The plants were watered three times each week with a balanced nutrient solution. Each week the crocks were thoroughly flushed with distilled water and nutrient solution applied immediately thereafter. The plants grew most rapidly at high temperature and humidity (chamber 1) and nearly as rapidly at high temperature and variable humidity (chamber 3), whereas in chamber 2 growth was much slower. The tops of the plants were supported with string in order that the foliage of each might receive as much light as possible. The plants were not pruned in any way. The differences between the plants in chamber 1 and chamber 3 were never very great, although the plants of chamber 1 had slightly longer internodes and somewhat longer leaves than those of chamber 3. The plants of chamber 3 more closely resembled comparable plants grown in the greenhouse than did plants of the other two chambers. The plants in chamber 2 grew much more slowly, produced short internodes and relatively large leaves. Growth of axillary buds was abundant, producing a plant with many branches and a bush appearance. Since these plants were used subsequently in another experiment no data are available as to dry weight accumulation and fruit weight.

Fruiting occurred at a much later date in chamber 2 than in the other two chambers, but the number of fruits set was larger. The fruits produced in the low temperature chamber were small and irregularly shaped (Watts, '31), whereas those at the higher temperatures were relatively small as compared to fruits produced in the greenhouse or out-of-doors, but were regular in shape. No differences were noted in the amount or appearance of fruits from chambers 1 and 3. The fruits were harvested on the morning of the day that they became red over the entire surface.

Ascorbic acid analyses were made on individual fruits. The results of the ascorbic acid analyses in milligrams per 100 gm. fresh weight were as follows: chamber 1, 18.5 ± 0.30 ($n = 29$); chamber 2, 15.6 ± 0.54 ($n = 34$); chamber 3, 19.4 ± 0.39 ($n = 11$); and from the greenhouse plants, 21.5 ± 0.35 ($n = 24$). The temperatures used in this experiment were fairly extreme. A continuous temperature of 63°F. is just about as low as tomato plants can stand to grow and still produce a crop, while a continuous temperature of 78°F. is approaching the upper temperature limits in which plants may be grown successfully. The ascorbic acid values at 63°F. (chamber 2) are significantly lower than those at 78°F. (chambers 1 and 3), although the differences are not great. The plants grown in the greenhouse produced fruit higher in ascorbic acid than in any of the chambers.

Length of photoperiod

Tomato plants of the Bonny Best inbred strain were grown in two of the control chambers in a manner very similar to that described in the previous section. The temperature of the two chambers was maintained at 73° ± 2°F., and the relative humidity varied from 60 to 85%. One chamber was illuminated for 8 hours each day and the other chamber for 16 hours. The experiment was conducted from April to July, and comparable plants were grown in the greenhouse, exposed to the natural variations in day length. The results of the ascorbic acid analyses in milligrams per 100 gm. fresh weight of fruit were as follows: chamber 1, 8-hour photoperiod, 16.7 ± 0.28 ($n = 53$); chamber 2, 16-hour photoperiod, 19.5 ± 0.33 ($n = 41$); greenhouse, 22.0 ± 0.37 ($n = 59$). The plants on the 8-hour photoperiod were significantly lower in ascorbic acid than those grown in the 16-hour photoperiod, whereas the plants grown in the greenhouse produced fruit appreciably higher in ascorbic acid than those from either of the chambers.

Sunshine and shade

On May 4, 1943, seeds of the Bonny Best inbred strain of tomatoes were planted in sand contained in small crocks (about five seeds per pot) and watered thrice weekly with a balanced nutrient solution. Once each week the pots were flooded with distilled water. Shortly after germination, the seedlings were thinned so as to leave two uniform seedlings per crock. On May 28, the seedlings were transplanted to quartz sand contained in 2-gallon crocks, one seedling per crock. On June 19, forty plants were placed outdoors in the sunshine and divided into groups of eight plants each. The individual plants of the various groups were randomized as to position.

One group of plants, designated as the sunshine control plants, remained in the sunshine throughout the rest of the experiment. The second group was grown in sunshine until the first blossoms appeared on June 22–23. These were tagged, and all unopened flower buds in that cluster were removed. The plants were then shaded with cheese cloth until the fruits ripened and were analyzed. The cheese cloth shade reduced illumination by 75% during bright days, as measured with a Weston foot-candle meter. The third group remained in sunshine until July 8, at which time each plant had three or four fruits, approximately ¼ of an inch in diameter. These were tagged; all other fruits and blossoms in that particular cluster were removed, and the plants placed in the shade where they remained until the fruits ripened and were analyzed. The fourth group was transferred to the shade on July 14, when each plant had three or four fruits approximately half grown, i.e., from 1 to 2 inches in diameter. The fifth group remained in the sunshine until each plant had developed from one to four "mature green" fruits, that is, of full size and pale green without any indication of red. Four plants of this group were transferred to shade on July 20, and four others on July 26.

A second lot of plants was handled in much the same way as that described above with respect to planting and cultural procedures. The seeds were planted on May 20 in the small crocks and transplanted to the larger crocks on June 10. On June 19, the plants were placed outdoors and exposed to full sunshine for 3 days. On June 22, when the plants were approximately 4 to 5 inches high, all plants were placed in the shaded area. Forty plants were used, and these were divided into five groups of eight plants each. The various groups were comparable to those previously described, except that the plants were transferred from shade to full sunlight at stages comparable to the transfer of the previous groups from sunshine to shade.

An outline of the treatments and vitamin analyses is given in table 4. Of the control plants, those grown throughout the experimental period with full exposure to sunshine produced fruits with an average ascorbic acid content of 25.8 ± 0.63 mg. per 100 gm. fresh weight, while those grown in shade produced fruits with an average ascorbic acid content of 15.5 ± 0.43 mg. The plants which were transferred from sunshine to shade produced fruits with an average ascorbic acid content not significantly different from the shade controls, regardless of the stage of development at the time of the transfer. The plants which were transferred from shade to sunshine produced fruit with an average ascorbic acid content not significantly different from the sunshine controls.

TABLE 4

Ascorbic acid content of tomato fruit from plants transferred from shade to sunlight and vice versa at various stages of fruit development.

STAGE OF FRUIT DEVELOPMENT AT TIME THE PLANTS WERE TRANSFERRED	TREATMENT OF PLANTS			
	Grown in sunshine[1]		Grown in shade[2]	
	No. of fruit analyzed	Ascorbic acid content in mg. per 100 gm. fresh wt.	No. of fruit analyzed	Ascorbic acid content in mg. per 100 gm. fresh wt.
Controls; not transferred	56	25.8 ± 0.63	48	15.5 ± 0.43
	Transferred to shade		Transferred to sunshine	
Transferred at time of first blossom	17	17.0 ± 0.88	27	26.1 ± 0.83
Transferred at time first fruits were ¼ in. in diameter	13	17.3 ± 1.75	19	27.0 ± 0.78
Transferred at time first fruits were ½ in. in diameter	27	15.8 ± 0.88	23	26.2 ± 0.68
Transferred at time first fruits were ''mature green''	23	16.8 ± 0.72	17	23.4 ± 1.08

[1] Plants were placed outdoors with full exposure to summer sunshine.

[2] Plants were placed under a cheese-cloth shade which resulted in decrease in illumination of about 75% during bright summer days (as measured with a Weston foot-candle meter).

It is apparent that the production of ripe fruit under shade has resulted in a considerable decrease in ascorbic acid content in comparison with fruit produced by plants growing in sunshine. The effect of shading seems to be produced during the period just before the fruits ripen.

No transfers were made at the time some of the fruits were partially colored, and therefore there was no measure of the rapidity with which a changing environment influences the ascorbic acid content during ripening.

DISCUSSION

The present study has dealt with the specific factors of possible significance in the variations in ascorbic acid content of tomatoes grown at different locations. Neither the nature of the soil nor fertilization has been found to exert any marked effect. There is also little evidence in this work that the location effects can be ascribed to differences in temperature, humidity, or variations in day length. The two temperatures used were fairly extreme. Plants growing in the field are subjected to temperature variations that are much more extreme but not continuous. It would seem that if temperature variations had a marked effect upon the ascorbic acid content of field-grown plants, greater differences would have been noted in the results of this experiment. It is possible that rapid fluctuations in temperature might have greater effects upon ascorbic acid values than continuous treatment with a low or a high temperature. The two day-lengths used were sufficiently different to warrant the expectation that much greater differences would not be observed under the natural variations in day length which occur from season to season. However, the illumination intensities used in the experimental chambers were relatively low, and it may be that at higher intensities, greater differences in ascorbic acid content would have been associated with the two day-length treatments. The two relative humidities used were sufficiently different to result in effects upon growth, but produced no effects upon ascorbic acid content. While much greater differences in humidity occur under field conditions, it seems likely that, were humidity playing a dominant role in determining ascorbic acid values, there would have been some differences in this experiment.

The one variable so far studied which seems to exercise marked effects is light intensity. Variations in ascorbic acid content associated with differences in this factor were relatively great. Furthermore, shifting the plants from one light intensity to another produced rapid changes in the ascorbic acid content of the fruit. It seems possible that the intensity of illumination may be the dominant factor in determining ascorbic acid values under many conditions.

Reports (Jones et al., '44) that nitrogen fertilization decreases ascorbic acid content of fruits seem conclusive. Our evidence indicates that this nitrogen effect may be an indirect one. It was found here that fertilization with nitrogen caused a decrease in ascorbic acid content of fruit from field-grown plants. In previous studies (Hamner et al., '42), no influence of nitrogen supply to tomato plants grown outside in sand culture was found. In the latter case, the plants were grown outside in sand cultures, the vines were supported on poles, and the axillary growth was removed. The pots were about 4 feet apart so that the individual plants were evenly illuminated by direct sunshine. In the fertilizer experiment reported here in which nitrogen fertilization decreased ascorbic acid content, the plants were grown in the field in rows 3 feet apart, and the plants were not pruned in any way. Thus, the heavy application of nitrogen in the field experiment undoubtedly produced more foliage, with resultant increased shading of some leaves by others. In the sand culture experiment on the other hand, while greater growth and more foliage were produced in the cultures supplied with more nitrogen, the design of the experiment was such that increased foliation did not cause increased shading. Thus, one might explain the effect of nitrogen in decreasing ascorbic acid content in one experiment and not in another.

Reports in the literature give abundant supporting evidence of the influence of light intensity on ascorbic acid content (Hamner and Maynard, '42; Maynard and Beeson, '43). This evidence includes reports that the amount of ascorbic acid in individual fruit on one tree may be proportional to the amount of light received during ripening. Cloudy weather has been reported to decrease ascorbic acid, and the side of individual fruit exposed to the sun has been found richer in ascorbic acid than the shaded side. There has been disagreement as to whether or not greenhouse-grown tomato fruits contain less ascorbic acid than field grown fruits, and it may be possible that those investigators who recorded the decrease in greenhouse-grown plants were utilizing greenhouses in which the intensity of illumination was appreciably lower than outdoors. In previous work from this laboratory (Hamner et al., '42), no difference between greenhouse and field-grown fruit was noted, but the plants grown in the greenhouse were exposed to intensities of illumination at least 90% of that outdoors.

Thus, the assumption that light intensity previous to harvest plays the dominant role in determining ascorbic acid levels in tomato fruit of a given variety does not seem to conflict with any of the other experimental data. It seems possible that much of the effect ascribed to season and location may be due to variations in this factor. Since this work indicated that rapid change in ascorbic acid takes place following a change in intensity of illumination, it would seem desirable to harvest fruit after a few days of bright sunny weather if maximum ascorbic acid values are to be obtained. It may be possible that production of fruit for canning purposes in regions where light intensity is consistently high would result in more ascorbic acid in the canned product, assuming, of course, that the processing methods preserved most of the ascorbic acid present in the fresh fruit. The results of this work emphasize the importance of considering illumination intensities in interpreting the results of experimentation dealing with factors influencing ascorbic acid content of plants.

SUMMARY

The influence of environmental variables on the ascorbic acid content of tomatoes has been studied in the field and under controlled conditions in the laboratory. Evidence is presented that ascorbic acid content is only slightly, if at all, influenced by: (1) degree of ripeness after the fruit is "mature green", (2) storage of the fruit for 10 to 14 days at temperatures from 65°F. to 90°F., (3) fertilizer treatment of the plants (although nitrogen fertilization may cause a slight decrease), and (4) relative humidity to which the plants are exposed.

Under controlled growing conditions fruits produced under lowered temperatures and shortened day length were somewhat lower in ascorbic acid.

By far the greatest influence on ascorbic acid content was produced by variations in light intensity previous to harvest. Increases in the ripe fruit of 66% in ascorbic acid resulted when plants were transferred from shade to sunshine at the time the fruit was mature green. A discussion is given of the possible significance of these results with the suggestion that the light intensity to which the plants are exposed just previous to harvest may be the dominant factor in determining the ascorbic acid content of ripe fruit.

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SHORT PERIOD BLOOD SUGAR TIME CURVES FOLLOWING INGESTION OF SUCROSE

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There is a widespread impression that, for immediate muscular work, the type of sugar is important; that dextrose is a better source of energy for this purpose than sucrose since, unlike the latter, it does not require digestion before it is absorbed from the alimentary tract. Many years ago, however, Higgins ('16) demonstrated the rapidity with which oral administration of sucrose may increase the respiratory quotient, as compared with dextrose; whereas, sucrose produced a very marked rise as early as 4 minutes, a definite rise was not noted with dextrose until a lapse of 20 minutes. The effects of other sugars and of alcohol were also investigated and, from these data, Higgins concluded that sucrose begins to be burned "quite as soon as alcohol". That sucrose affects the respiratory quotient more rapidly than dextrose was also noted later by Deuel ('27) and more recently by Carpenter ('40). Higgins suggested that part of the increase of the respiratory quotient following ingestion of sucrose, especially when the quotient exceeds 1.0, is due to formation of fat. Lactic acid formation from the fructose fraction, with resultant liberation of carbon dioxide, has been suggested as a factor (Cathcart and Markowitz, '27; Campbell and Maltby, '28; Wierzuckowski and Laniewski, '31; Bachmann and Haldi, '37). Edwards, Bensley, Dill and Carpenter ('44), however, have clearly shown that after allowing for the effects of blood lactic acid, the corrected quotient was still greater after fructose than after the same amount of glucose. Regardless of the cause — combustion or synthesis — the relevant facts here are that the respiratory quotient increases rapidly; that it increases more rapidly than with dextrose, and that sucrose cannot produce such increase until the products of its hydrolysis have been absorbed from the alimentary tract.

Diabetics who require insulin and who suffer from hypoglycaemic reactions as the result of overdosage afford a unique opportunity for testing the rapidity with which sucrose is hydrolyzed in the intestinal tract and the rapidity with which the product of this hydrolysis are absorbed by observation of (a) the response to sucrose ingestion subjectively and (b) the rapidity with which the ingested sucrose increases the concentration of sugar in the blood.

It is a well-known fact that, though the hypoglycaemia produced by an overdose of insulin may continue for hours in severe cases and require large amounts (hundreds of grams) of sugar to combat it, even when the latter is administered intravenously, in very mild cases the disagreeable symptoms may disappear within 2 or 3 minutes — in some cases in 1 minute — following ingestion of a little orange juice, provided the latter is taken on an empty stomach. For this reason, it has been a routine practice in the wards of The Montreal General Hospital since insulin was first used to treat all mild reactions in this manner. Oranges, however, in their natural state, contain approximately equal quantities of sucrose and dextrose (Roberts and Gaddum, '37). No conclusions, therefore, can be drawn from these experiences with respect to the rapidity with which sucrose increases the blood sugar. The experiences with diabetics in their homes, however, are of some significance. It has been the routine during the same period to advise such patients to carry with them at all times the ordinary commercial cubes of cane sugar, and the results have been equally satisfactory; dextrose has never been used in this clinic for the control of insulin reactions, except in very severe cases which required sugar intravenously Subjective improvement, however, is not as reliable as actual determination of the sugar content of the blood as an indication of the rapidity with which sugars or their derivatives are absorbed from the alimentary tract The purpose here, therefore, is to record the results of blood sugar determinations made in cases in insulin hypoglycaemia at 1-minute intervals following ingestion of sucrose.

METHOD OF INVESTIGATION

Selection of subjects

With large excesses of insulin, no increase of blood sugar may be noted for many hours even after administration of large amounts (hundreds of grams) of sugar (Rabinowitch, Fowler and Bensley, '37). Such cases, therefore, afford no indication of the rapidity with which the administered sugar is absorbed from the alimentary tract. The subjects used in this investigation, therefore, included only those who had very mild reactions.

Condition of subjects

Failure to note a rapid increase of blood sugar following ingestion of sugar may be due not to slow absorption directly but to a slow emptying of the stomach; it is well known, that food leaves the stomach most rapidly when it is administered on an empty stomach. In order, therefore, to exclude as much as possible artificially delayed absorption due to this factor, the only subjects studied were those who had insulin reactions in the fasting state, that is, in the morning, approximately, 15 hours after the last (evening) meal. Without exception, all of the subjects were being treated with protamine zinc insulin which, because of its prolonged action, tends to produce low blood sugars the following morning in the fasting state — 24 hours after its injection.

Sucrose drink

Aside from the degree of emptiness of the stomach, other conditions which effect the emptying time of the stomach are (a) the fluidity of the meal and (b) its osmotic pressure: the more liquid the meal, the sooner does it leave the stomach; hypotonic solutions leave the stomach more rapidly than isotonic solutions. In order, therefore, to avoid artificially delayed absorption of the ingested sugar due to these factors, the sucrose was administered in aqueous solution, and the latter was made hypotonic (5%).

Though the amount of sugar influences the time during which an increase of blood sugar may be prolonged, the height of the response is not proportional to the amount of sugar administered (MacLean and de Wesselow, '20–'21; Hansen, '23; Tisdall, Drake and Brown, '25). All subjects were, therefore, given the same amount, namely 10 gm.

Venous vs arterial blood sugars

In a normal person, after ingestion of a sugar, the arterial and venous blood sugars are equally affected until the blood sugar reaches a level of about 0.120%. Thereafter, for a time, the arterial blood sugar rises to a higher level than the venous (Foster, '23; Rabinowitch, '27). Therefore, since a normal person might have a blood sugar close to 0.120% before ingestion of sugar, analysis of the venous blood may fail to detect the beginning of absorption of the ingested sugar. Determination of true arterial blood sugar, however, involves radial artery or other arterial puncture which, at 1-minute intervals, is not practical. In cases of hypoglycaemia, however, where the blood sugars are well below 0.120% venous blood is as satisfactory as arterial. All tests were, therefore, made on venous blood.

Routine procedure

In each case, the concentration of sugar in the blood was determined before and then at 1-minute intervals after ingestion of the sucrose drink until the patient stated he "felt better".

As stated, with sufficiently large excesses of insulin, no change whatever may be noted in the blood sugar for many hours even in spite of absorption of hundreds of grams of sugar. The only subjects of interest here, therefore, were those in whom subjective improvement was noted very soon after the ingestion of the sucrose drink.

In the interpretation of the data, evidence of absorption of sugar was not regarded as positive unless the increase of blood sugar was in excess of 10 mg. per 100 ml. which is well in excess of the experimental error of the test used for the determination of the blood sugar. Blood sugar was determined by the Myers and Bailey ('16) modification of the Lewis-Benedict macro method, with all reagents crystallized repeatedly and the color determined by a colorimeter or by a photo-electric cell. The combined data of ten experiments (eight subjects) are shown in table 1.

DISCUSSION OF RESULTS

With three exceptions (experiments no. 2, 5 and 6), it will be noted that all bloods were markedly hypoglycaemic before the ingestion of the sucrose. The cases were, therefore, properly selected with regard to the reliability of venous blood sugars as an indication of the beginning of absorption of sugar from the alimentary tract.

No relationship was noted between the degree of hypoglycaemia and subjective disturbances — all reactions were mild — fitting in with previously reported experiences (Peters and Rabinowitch, '29; Rabinowitch, Fowler and Corcoran, '36) and now a well recognized phenomenon.

In all of the cases, subjective improvement was noted within 5 minutes of the ingestion of the sucrose, and it will be noted that of the ten experiments a definite increase of blood sugar (that is, in excess of 10 mg. per 100 ml. of blood) was noted within 1 minute in two cases, within 2 minutes in two cases, within 3 minutes in three cases, within 4 minutes in two cases and within 5 minutes in the remaining case.

A possibility which had to be considered was "emotional" hyperglycaemia due to discomfort from the needle punctures, that is, an increase of blood sugar not due to absorption of sugar from the alimentary canal, but to mobilization of sugar from its stores in the body. Though such hyperglycaemia undoubtedly occurs with marked pain and fear (Bohm and Hoffmann, 1878; Cannon, Shohl and Wright, '11; Griffith, '23; Britton, '28) the writer, from experiences with thousands of blood sugar time curves, has never been impressed with the emotional effects of single needle punctures, provided the needle was sharp and each sample of blood was collected by an experienced person requiring one needle puncture only. In this study, however, unlike the ordinary blood sugar time curves for which blood samples are taken at ½- and 1-hour intervals, blood samples were collected at 1-minute intervals. To exclude "emotional" hyperglycaemia as much as possible, therefore, particular care was taken with regard to the sharpness of the needles and all samples of blood were collected by the writer. As a control, however, in three experiments (nos. 8, 9 and 10) samples of blood were collected at 1-minute intervals for 5 minutes before the administration of the sucrose drink. The blood sugar data so obtained are shown in table 2, from which it will be noted that emotional hyperglycaemia was not a factor in the production of the increases of blood sugar noted soon after the ingestion of the sucrose drink. Fluctuations of the blood sugar were noted, but, without exception, all were slight and within the range of the experimental error of the test for the determination of blood sugar, i.e., less than 10 mg. per 100 ml. of blood.

TABLE 1

Short period blood sugar time curves following oral administration of sucrose.

NO.	BLOOD SUGAR					
	Fasting state	Minutes after ingestion of sucrose				
		1	2	3	4	5
	%	%	%	%	%	%
1	0.056	0.050	0.066	0.103	0.133	
2	0.068	0.066	0.065	0.083	0.125	
3	0.063	0.060	0.064	0.065	0.063	0.111
4	0.045	0.044	0.084	0.093	0.129	
5	0.066	0.064	0.067	0.070	0.080	0.122
6	0.069	0.083	0.120			
7	0.040	0.073	0.083	0.125		
8	0.053	0.051	0.066	0.073	0.080	0.123
9	0.058	0.061	0.055	0.082	0.099	
10	0.045	0.046	0.046	0.053	0.068	0.100
A.M.	0.056	0.060	0.072	0.083	0.097	0.114

Note: No. 4 and 7 same subject 3 days apart. No. 6 and 8 same subject 11 days apart.

The rapid increases of blood sugar noted after ingestion of sucrose in these experiments fit in with the above-mentioned rapid increase of the respiratory quotient. The data for both the respiratory quotients and blood sugar indicate clearly that sucrose is very rapidly hydrolyzed and that the products of its hydrolysis are very rapidly absorbed. The impression, therefore, that dextrose is superior to sucrose as an immediate source of energy because it requires no preliminary digestion is without foundation. In fact, there is reason, experimentally and theoretically, to believe that sucrose, because of its laevulose fraction, is the superior sugar. Its apparently superior protein-sparing and ketolytic actions (Deuel, Gulick and Butts, '32) and its greater ability to maintain the blood sugar concentration during exercise (Dische and Goldhammer, '32) are examples. Whatever the mechanism may be, the fact of practical importance, and clearly demonstrated here, is that, though sucrose must be hydrolyzed before it is available as a source of energy, the hydrolysis is almost "explosive" in character and thus does not delay the availability of the sugar.

TABLE 2

Short period blood sugar time curves in the absence of sugar administration.

SUBJECTS IN TABLE 1	BLOOD SUGAR					
	Before venous puncture	After venous puncture (minutes)				
		1	2	3	4	5
	%	%	%	%	%	%
8	0.051	0.049	0.056	0.050	0.054	0.053
9	0.053	0.059	0.056	0.061	0.060	0.058
10	0.040	0.046	0.045	0.044	0.049	0.045
A.M.	0.048	0.051	0.052	0.052	0.054	0.052

SUMMARY

Blood sugar time curves were obtained at 1-minute intervals following ingestion of sucrose, in order to determine the rapidity with which this food is available as a source of energy, using the increase of blood sugar as the indication of absorption from the alimentary tract.

Of the ten experiments reported here, a definite increase of blood sugar was noted within 1 minute in two cases, within 2 minutes in two cases, within 3 minutes in three cases, within 4 minutes in two cases and within 5 minutes in the remaining case. "Emotional" hyperglycaemia was excluded as a possible cause of the increase of sugar noted in three experiments.

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NEUROPATHOLOGIC STUDIES OF PANTOTHENIC ACID, BIOTIN AND FOLIC ACID COMPLEX DEFICIENCIES IN THE CHICK[1]

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The paralysis and neuropathologic lesions in pantothenic acid deficiency in the chick have been described by Phillips and Engel ('39). This deficiency was produced on the heated ration of Mickelsen, Waisman and Elvehjem ('38) who have described the dermatitis which occurred. The former authors found that in chicks fed the heated ration, there was a severe degeneration of the myelin and axons of the spinal cord and occasionally of the peripheral nerves. They found that pantothenic acid was necessary for the prevention of the neuropathologic lesions in the spinal cord and that riboflavin was necessary to prevent those in the peripheral nerves. Since that time, it has been shown by Waisman, Mills and Elvehjem ('42) that the heated ration was also deficient in other factors required in the nutrition of the chick. Due to these complicating factors, it seemed wise to reinvestigate the pathology of pantothenic acid deficiency in the chick as produced on the heated ration and on a complete sucrose ration.

EXPERIMENTAL

The heated ration used was the modification of the heated diet used by Waisman, Mills and Elvehjem ('42). It had the following percentage composition: ground yellow corn, 56; standard wheat middlings, 25; crude casein, 12; soybean oil, 3; and a salt mixture, 4. The chicks were given 2 drops of halibut liver oil weekly. Although the original workers heated the grain and casein portion of the ration for 100 hours at 100°C. to destroy pantothenic acid, later work showed that

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a 30-hour treatment of the ration (Mickelsen, Waisman, and Elvehjem, '38) at 120°C. gave a more consistent incidence of dermatitis and paralysis.

In this series of experiments each 100 gm. of the basal ration was supplemented with 300 μg. of thiamine, 400 μg. of riboflavin, 400 μg. of pyridoxine, 100 mg. of choline, and 0.50 mg. of 2-methyl, -1, 4-naptho-quinone. There was no growth stimulation when these were added to the basal ration, but the chicks did survive for a longer period.

Since the rate of growth was very far below the maximum even when an ample amount of pantothenic acid was added to the basal ration, various supplements were added to improve the growth and to study the effect on the accompanying pathologic lesions. Incidentally, it was possible to study the pathologic changes in a mild type of biotin deficiency which developed when 10% cartilage and 3% solubilized liver fraction were added to the basal heated ration supplemented with 15 mg. of calcium pantothenate per kilogram.

In order to study the neuropathologic lesions of pantothenic acid deficiency, uncomplicated by other factors, in contrast to the chicks fed the supplemented heated ration, a sucrose ration was used. Its percentage composition was as follows: sucrose, 54; kidney residue, 3; cartilage, 15; alcohol-extracted casein, 18; salts IV [2] 5, and soybean oil 5, and a factor concentrate equivalent to 10% of yeast. One hundred gm. of the basal ration were supplemented with 300 μg. of thiamine, 400 μg. of riboflavin, 400 μg. of pyridoxine, 100 mg. of choline, and 10 mg. of nicotinic acid. The chicks were given 2 drops of halibut liver oil weekly. Supplements of calcium pantothenate of from 2 to 20 mg. per kilogram were added to study the rate of alleviation of the pantothenic acid deficiency.

In order to eliminate the possibility of a deficiency of the folic acid complex being involved in the pantothenic acid deficiency produced on either the heated or the sucrose basal rations, deficiency of the folic acid complex was produced by the use of the following ration: dextrin, 56.7%, alcohol extracted casein 18%, salts IV 5%, $CaHPO_4$ 1%, soybean oil 5%, liver residue 4%, gelatin 10%, and cystine 0.3%. This ration was supplemented with thiamine, riboflavin, pyridoxine, choline, nicotinic acid, and halibut liver oil in the same amounts as in the basal ration itemized in the preceding paragraph.

The deficient chicks were autopsied when they became so severely deficient that death was imminent. The corresponding controls were simultaneously sacrificed for comparison.

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[2] Phillips and Hart (J. Biol. Chem., vol. 109, p. 657, 1935).

The sciatic nerves were studied by means of the polarizing microscope (Setterfield and Sutton, '35), the Marchi osmic acid reaction (Swank and Davenport, '34) and by the silver impregnation method of Bodian ('36). The spinal cords were studied after they were stained by Einarson's method ('32), by the Marchi reaction and by the silver impregnation method of Bodian ('36).

RESULTS

On the heated ration only, there was a severe myelin degeneration in the spinal cord in all four chicks. When 15 mg. of calcium pantothenate were added to the basal ration, no paralysis was observed and there were no pathologic lesions in the spinal cord of the six chicks. When a solubilized liver fraction supplement at a 3% level was added as a source of the folic acid complex, there was a very appreciable increase in the rate of growth. When a supplement of 10% cartilage or a supplement of 3% glycine, 0.5% arginine and 0.5% cystine was added as a source of the cartilage factor, there was a further increase in growth. In both of these latter groups, a very pronounced biotin deficiency was present as evidenced by the presence of a dry scaly callous condition on the bottom of the feet and fissuring between the toes (Hegsted, Oleson, Mills, Elvehjem and Hart, '40, and Hegsted, Mills, Briggs, Elvehjem and Hart, '42). It was found that there was no evidence of any lesion in the spinal cord or in the sciatic nerves of these chicks that were mildly deficient in biotin.

On the sucrose ration which was complete in all known factors except for pantothenic acid, dermatitis and paralysis were observed in all six chicks. The same type of myelin and axon degeneration was found in the spinal cord as was present in the chicks which developed paralysis on the heated ration. When 2 mg. of calcium pantothenate were added per kilogram of ration, there was still a severe degree of myelin degeneration in the spinal cords of the four chicks in the group. When 3 mg. of calcium pantothenate were added per kilogram, the lesions in the spinal cord were still quite pronounced in all four chicks. However, a supplement of 5 mg. of calcium pantothenate per kilogram prevented all but a slight trace of the myelin degeneration. Levels of 10 and 20 mg. of calcium pantothenate per kilogram completely prevented the neuropathologic lesions. There was no case in which any myelin degeneration was observed in the sciatic nerve.

The lesions in the spinal cord were characterized by a widespread myelin degeneration in all areas of the white matter. This occurred to the greatest extent in the lateral and anterior columns and extended from the cervical region to the lumbar region of the cord. The axons in these regions frequently showed mild degenerative changes although not to the same extent as the accompanying myelin degeneration. Some very mild changes were observed in the Nissl materials of the nerve cells in the cervical and brachial regions of the spinal cord.

The chicks on the ration deficient in the folic acid complex did not have any of the incrustations about the beak and feet that occurred in pantothenic acid deficiency nor the callousness and fissuring of the feet that occurred in biotin deficiency. The chicks were not too well feathered. After 3 or 4 weeks on the ration they became so weak as to be unable to stand. They would rest on the floor of the cage and when they were disturbed, they seemed to be unable to get up on their feet. They appeared to have a good sense of balance but were too weak to support their own weight.

No lesions were found in the spinal cord of the seven deficient chicks. In two of these chicks there was a very mild myelin degeneration in the sciatic nerve apparently unaccompanied by any axon change. It was found impossible to keep these chicks alive once they became so weak that they could not support their own weight. It was thus impossible to determine if these occasional lesions in the sciatic nerve would become more severe in a prolonged deficiency. No lesions were found in the nerve tissues or the control groups which received 2% solubilized liver fraction or a folic acid concentrate equivalent to 5% solubilized liver fraction.

DISCUSSION

The neuropathologic lesions of pantothenic acid deficiency were studied in chicks on the heated ration supplemented with the crystalline vitamin B complex vitamins and on a sucrose ration which was believed to be otherwise complete. The pathological changes in the spinal cord, which were reported previously by Phillips and Engel ('39) in chicks on the unsupplemented heated ration and which were prevented by pantothenic acid, were seen on both of these improved rations. The deficiencies which were still present in the supplemented heated ration did not complicate the picture of the spinal cord lesions in the pantothenic acid deficient chicks. On the heated ration, the primary and most severe deficiency was that of pantothenic acid. Not until sufficient pantothenic acid was supplied did the effect of biotin and folic acid complex deficiencies appear. A study of the mild biotin deficiency and of an acute deficiency of the folic acid complex revealed no neuropathologic changes. Therefore, it was concluded that the myelin and axon degeneration observed in the spinal cord in pantothenic acid deficiency in the chick was due to the deficiency of pantothenic acid alone and not to the lack of any other nutritional factor.

The fact that myelin and axon degeneration occurred in the spinal cord and not in the peripheral nerves of the pantothenic acid deficient chick is unexplained. In both thiamine and riboflavin deficiencies, in chicks, the first degeneration of myelin to be observed was in the peripheral nerves and not in the spinal cord. If the spinal cord becomes involved at all in these cases it is only much later in the progress of the deficiency. However, in pantothenic acid deficiency the spinal cord alone was involved. The areas of the spinal cord which were involved in pantothenic acid deficiency were the lateral and anterior columns. These were usually free of any degeneration in a riboflavin deficiency.

SUMMARY

1. The deficiency of pantothenic acid in the chick caused a very widespread myelin and axon degeneration in the spinal cord. There was no accompanying degeneration in the peripheral nerves.
2. These lesions of the spinal cord occurred in pantothenic acid deficiency, whether it was produced on a very incomplete ration such as the heated ration or on an otherwise complete sucrose ration.
3. Complicating deficiencies which appeared on the heated ration did not seem to alter the neuropathologic lesions in a pantothenic acid deficiency.
4. No neuropathologic lesions were observed in chicks suffering from a mild biotin deficiency, or an acute deficiency of the folic acid complex.

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NEUROPATHOLOGIC STUDIES OF ACUTE AND CHRONIC THIAMINE DEFICIENCIES AND OF INANITION[1]

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TWO TEXT FIGURES AND ONE PLATE (NINE FIGURES)

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Prickett, Salmon and Schrader ('39) observed differences between the pathological changes in acute and chronic thiamine deficiencies. Rats with acute thiamine deficiency had only a few fibers that showed Wallerian degeneration. However, nerves of deficient rats, that became moribund without any evidence of the neuromuscular symptoms which are typical of the acute stage of the deficiency, showed a much more extensive myelin and axon degeneration. Zimmerman ('39, '40) emphasized the difference between the acute and chronic states of thiamine deficiency in the pigeon, rat and dog. Street, Zimmerman, Cowgill, Hoff and Fox ('41) further discussed the effects of a long-continued subminimal intake of thiamine. In dogs, they observed a moderate spasticity of the hind legs, unsteadiness, staggering and vomiting, with the absence of the deep reflexes of the hind legs. Histological studies of the nervous system revealed an extensive myelin degeneration of both the peripheral nerves and the posterior columns of the spinal cord. These lesions were observed on a diet which contained 8% autoclaved yeast. Swank ('40) also reported appreciable differences in the neuropathology of pigeons in acute and chronic thiamine deficiencies.

Several authors have discussed the effect that the inanition, which always occurs early in the thiamine deficiency, has on the pathology observed. The need for consideration of this complicating factor is a real one, especially in those cases where a prolonged chronic deficiency is produced. Prickett, Salmon and Schrader ('39) and Street, Zimmerman, Cowgill, Hoff and Fox ('41) reported myelin degeneration in the

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We are indebted to Merck and Co., Rahway, New Jersey, for supplies of crystalline vitamins and to Abbott Laboratories, North Chicago, Illinois, for halibut liver oil and Klotogen.

peripheral nervous system of the pigeons, dogs and rats which had received a restricted intake of a ration containing ample thiamine. By this means they simulated a starved condition similar to that developed by chronically thiamine deficient animals. These authors stated that the changes were less severe and less frequent in starved, than in chronically thiamine deficient animals.

Swank ('40) reported that the blackening of myelin sheaths by the Marchi method was not pathognomic of degeneration. He stated that the blackening which occurred in the peripheral nerves of starved pigeons was not truly a myelin degeneration.

Due to the great confusion in the literature which has arisen because of the different degrees of thiamine deficiency studied by various workers and the complicating factor of inanition, it was decided to study the pathology of inanition and of acute and chronic thiamine deficiencies, as nearly as possible uncomplicated by any other nutritional deficiency.

EXPERIMENTAL

The first experiment was done with 6- to 8-week-old pigeons in a manner similar to that described by Swank ('40). Preliminary trials were made with Swank's diet II the percentage composition of which was corn starch 66, alcohol-extracted casein 20, cod liver oil 4, peanut oil 6, and salts IV [2] 4. This ration was supplemented with a vitamin K concentrate [3] at a level equivalent to 3 Almquist units per pigeon per day. After preliminary trials, this ration was changed considerably, in order (1) to make it more suitable for feeding by pipette, (2) to improve its digestibility, thus reducing weight loss, and vomiting, but increasing the survival time, and (3) to reduce the fat level in order to make the ration less thiamine-sparing. The corn starch was replaced by dextrin, the peanut oil was reduced from 6 to 4%, and the cod liver oil left out of the ration. The basal ration then consisted of: dextrin 72%, casein 20%, peanut oil 4%, and salts IV 4%. It was supplemented with adequate irradiated ergosterol, shark liver oil, and the vitamin K concentrate.

This ration was mixed with enough distilled water to make it sufficiently fluid to allow it to be pipetted through a 50 ml. quick-delivery pipette. This suspension of the ration was made daily. The pigeon was held by a strip of Gooch rubber tubing which was snugly wrapped around its breast and wings and fastened with a hemostat. By this means, it was possible to immobilize the bird without interfering with its normal breathing, until a known amount of ration suspension could be introduced into its crop.

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[2] Phillips and Hart (J. Biol. Chem., vol. 109, p. 657, 1935).

[3] Abbott's Klotogen.

The pigeons of the first group were fed only 4 gm. of the basal ration daily, in order that they would be starved as well as rendered acutely deficient in thiamine. Those in the second group were fed 10 to 15 gm. of the basal ration per day, enough to prevent complication of the acute deficiency by starvation effects. Those in the third group were fed 10 to 15 gm. of the basal ration but were given a minimal daily injection of thiamine intramuscularly to produce a chronic thiamine deficiency, supposedly uncomplicated by any starvation effects. Those in the fourth group were given 4 gm. of the basal ration plus a daily injection of 100 μg. of thiamine, in order that they would be starved but not deficient of the vitamin. The pigeons in the fifth group were given ample ration plus 100 μg. of thiamine daily.

Each of the five groups was subdivided into three subgroups. The pigeons in subgroup A of each group were given the basal ration. Those in subgroup B were given the basal ration, supplemented with 100 μg. of riboflavin and 0.5 mg. of pantothenic acid per day. Those in subgroup C received the basal ration, supplemented daily with a gelatin capsule containing 100 μg. of riboflavin, 100 μg. of pyridoxine, 0.5 mg. of pantothenic acid, 15 mg. of choline, 15 mg. of inositol, 1 mg. of nicotinic acid, 15 mg. of para-aminobenzoic acid and 0.5 gm. of a sulfite treated 1:20 liver extract.

Day-old chicks were used in the second experiment. They were found to be more satisfactory animals, since a complete ration was available which could be made stringently deficient in thiamine by the omission of crystalline thiamine. Ten groups of chicks were used as outlined below.

The basal ration used in this experiment consisted of: dextrin 54.7%, alcohol-extracted crude casein 18%, gelatin 10%, salts IV 5%, $CaHPO_4$ 1%, soy bean oil 5%, liver residue 4%, solubilized liver extract 2%, and cystine 0.3%. This mixture was supplemented with crystalline vitamins at the following levels per kilogram: riboflavin 6 mg., pyridoxine 4 mg., pantothenic acid 15 mg., choline 1.5 gm., and nicotinic acid 100 mg. Two drops of halibut liver oil were given weekly to each chick. This ration, when supplemented with 4 mg. of crystalline thiamine per kilogram, gave good growth in chicks. Since it was impossible to feed day-old chicks by pipette, it was necessary to allow them to consume the ration ad libitum.

The chicks in group 1 were fed the basal ration to produce an immediate, acute thiamine deficiency. Those in group 2 were first given the basal ration plus a 20 μg. injection of thiamine daily for 2 weeks. Then an acute thiamine deficiency was allowed to develop.

The chicks in group 3 were fed the basal ration until symptoms of an acute deficiency began to appear. Then subminimal injections of thiamine were given daily in order to prevent death and to alleviate partially the opisthotonus of the acute deficiency. The injections were continued until all signs of the acute deficiency had disappeared and the chicks had become chronically paralyzed. Those in group 4 were given the basal ration plus 20 μg. of thiamine daily for 2 weeks only. When the acute symptoms began to appear, subminimal injections of thiamine were given as in group 3. In groups 5, 6, 7, and 8, the chicks were treated like those in group 4 except that the following daily injections were given: 20 μg. of riboflavin to those in group 5, 100 μg. of pantothenic acid to those in group 6, 20 μg. of pyridoxine to those in group 7, and 20 μg. of riboflavin, 100 μg. of pantothenic acid, and 20 μg. of pyridoxine to those in group 8.

The chicks in group 9 were fed the basal ration plus an injection of 40 μg. of thiamine per day. Their daily intake was limited to that of individual chicks in the chronic thiamine deficient groups. Those in group 10 were allowed to consume the basal ration ad libitum and were given a thiamine injection of 40 μg. per day.

In each of these experiments, when the deficient or starved pigeons and chicks became so severely affected that death was imminent, they were killed and autopsied. Controls, which had received ample thiamine were sacrificed and autopsied in the same manner. The brain, the brachial segment of the spinal cord, one brachial and sciatic nerve were fixed in a solution consisting of: 95% ethanol 100 parts, glacial acetic acid 5 parts, and paraldehyde 2 parts. After dehydration, imbedding and sectioning, these tissues were stained by the Bodian silver technique ('36). A cervical portion of the spinal cord, one brachial, one sciatic and the nerve to the pectoralis muscle were fixed in a 10% formalin solution buffered at a pH of 7.0. The spinal cord and one-half of the brachial and sciatic nerves and the nerve to the pectoralis muscle were stained, by a modified Marchi osmic acid method (Swank and Davenport, '34). The other half of the brachial and sciatic nerves were used for polarizing microscope studies of the birefringent materials between crossed Nicols (Setterfield and Sutton, '35).

In order to study the pyruvic acid metabolism of these thiamine deficient chicks, pyruvic acid analyses were made of the blood, sciatic nerve and gastrocnemius muscle. The method used for these analyses was that of Bueding and Wortis ('40).

RESULTS

Pigeons

The observations of the pigeons are reported in table 1. In group 1 (acutely thiamine deficient and starved) there was a mild myelin and axon degeneration in the sciatic and brachial nerves of six of the ten pigeons and a mild myelin and axon degeneration in the spinal cord of one of them. A daily supplement of all the known crystalline members of the vitamin B complex did not appear to alter the combined effects of the acute thiamine deficiency and the starvation.

In group 2 (acutely thiamine deficient but not starved), a mild myelin degeneration was observed in the peripheral nerves of six of the eleven pigeons. There was also a mild myelin degeneration of the spinal cord in four of them. However, these changes were of a minor nature and there was only slight axon degeneration in the peripheral nerves and the spinal cord. The vitamin supplements did not prevent any of the lesions.

In group 3 (chronically thiamine deficient but not starved), a very definite myelin and axon degeneration was observed in the peripheral nerves of thirty-seven of the thirty-eight pigeons. There was some degeneration in the spinal cord (fig. 11) but this was neither as frequent nor as severe as that observed in the peripheral nerves (figs. 8 and 10). The degenerated fibres in the spinal cord were not localized in any definite region. The posterior columns, however, were almost completely free of any degenerative changes. The leg weakness that developed in these pigeons was never comparable in extent to that seen in the corresponding chicks in experiment 2 which is described later.

The chronically thiamine deficient pigeons did not readily digest the ration which was introduced into their crops. As they became more chronically deficient, there was a gradual reduction in the amount of food which they could pass into the intestinal tract from the crop. Atony of the crop appeared to develop and the purpose of forced feeding was in this way defeated. The digestive system of these pigeons seemed capable of handling only a very small amount of ration, approximately comparable to that actually eaten by a pigeon that is chronically deficient.

In group 4 (starved but ample thiamine), there was observed a mild myelin degeneration of the peripheral nerves and spinal cord comparable to that observed in group 1. The vitamin supplements and sulfited 1:20 liver extract seemed to have no preventative effect on the development of the lesions.

TABLE 1

Birds which developed histopathological changes in the spinal cord and sciatic nerve.

GROUP NO.	SUB-GROUP	RATION	TYPE OF DEFICIENCY	NO. OF BIRDS	NO. OF BIRDS SURVIVING	SPINAL CORD	SCIATIC NERVE
		Experiment 1 — Pigeons					
1	A	Basal only — restricted intake	Acute and starved	4	4	1	2
	B	A + 100 μg. riboflavin + 0.5 mg. pantothenic acid/day	Acute and starved	4	3	0	2
	C	A + vitamin B complex mixture	Acute and starved	4	3	0	2
2	A	Basal only — ample	Acute	4	4	1	2
	B	A + 100 μg. riboflavin + 0.5 mg. pantothenic acid/day	Acute	4	4	1	2
	C	A + vitamin B complex mixture	Acute	4	3	2	2
3	A	Basal — ample + subminimal thiamine	Chronic	12	11	3	11
	B	A + 100 μg. riboflavin + 0.5 mg. pantothenic acid/day	Chronic	20	19	9	18
	C	A + vitamin B complex mixture	Chronic	12	8	5	8
4	A	Basal + 100 μg. B_1/day — restricted intake	Starved	8	8	3	4
	B	A + 100 μg. riboflavin + 0.5 mg. pantothenic acid/day	Starved	12	9	2	2
	C	A + vitamin B complex mixture	Starved	8	5	0	2
5	A	Basal + 100 μg. B_1/day — ample ration	None	4	3	0	1
	B	A + 100 μg. riboflavin + 0.5 mg. pantothenic acid/day	None	4	3	0	1
	C	A + vitamin B complex mixture	None	4	4	1	1
		Experiment 2 — Chicks					
1		Basal only	Acute	5	5	0	0
2		Basal + 20 μg. B_1/day for 2 weeks — then basal only	Acute	14	14	1	2
3		Basal + subminimal injection of B_1 daily	Chronic	10	5	3	5
4		Basal + 20 μg. B_1/day for 2 weeks — then subminimal injection of B_1 daily	Chronic	30	26	8	25
5		4 + 20 μg. riboflavin/day	Chronic	17	15	6	14
6		4 + 100 μg. pantothenic acid/day	Chronic	17	13	5	12
7		4 + 20 μg. pyridoxine/day	Chronic	17	15	5	15
8		4 + 20 μg. riboflavin + 100 μg. pantothenic acid + 20 μg. pyridoxine/day	Chronic	17	12	4	11
9		Basal + 40 μg. B_1/day restricted intake	Starvation	13	13	5	13
10		Basal + 40 μg. B_1/day ad libitum	None	14	14	0	0

The pigeons of group 5 (ample thiamine and ample ration) showed no pathological lesions (fig. 3) except in a few isolated cases. The ration was still so deficient that no growth was observed although those in subgroup C did maintain their original weight.

Chicks

The results observed in the chicks in experiment 2 are recorded in table 1. In the chicks of group 1 subjected to an immediate, acute thiamine deficiency, the first acute deficiency signs appeared after 8 or 9 days. Typical head retractions had developed by that time and the chicks were dead within 24 hours after the onset of these symptoms. No definite pathology could be seen in either the sciatic nerve (figs. 5 and 6) or the spinal cord. In group 2 where an acute deficiency was produced after 2 weeks of normal growth, opisthotonus appeared 7 to 10 days after the daily injections of thiamine had been discontinued. Again, the acutely deficient chicks died within 24 hours after the onset of the visible symptoms. In two of the fourteen chicks autopsied, a slight speckling was observed in the myelin of the sciatic nerve and of the spinal cord; the remainder appeared to be completely normal. The changes observed in these two chicks were not typical of any described stage of myelin degeneration.

In group 3, a chronic thiamine deficiency was produced in only five of the ten chicks; the others died in a more acute stage. Due to the sensitivity of the young chick to thiamine deficiency, it was very difficult to keep them alive once the symptoms of acute thiamine deficiency became evident. In each case the chronic deficiency was not a prolonged one. Five of these chronically deficient chicks showed a mild myelin degeneration in the sciatic nerve; and three of these had a very mild degeneration in scattered areas of the spinal cord.

When the chicks were allowed to grow normally for 2 weeks, as in groups 4–8 inclusive, it was possible to produce a chronic thiamine deficiency more readily. This was done by the injection of a subminimal dose of 2–4 µg. of thiamine daily. In these chicks which weighed an average of 110 gm. at the appearance of deficiency symptoms, these symptoms could be alleviated by this small daily injection. However, enough thiamine to permit a complete recovery was never given. Thus, the chicks could be maintained in a state of chronic thiamine deficiency for as long as 3 weeks. A very noticeable and severe leg paralysis developed after 4–7 days in this chronic state. In the early stages of the paralysis (fig. 1), the toes were curled inwards slightly. As the chronic condition was prolonged, the "curled toe" condition was replaced by a rigid extension of the legs (fig. 2). In almost every case in groups 4–8 where a chronic thiamine deficiency was produced, there was a very definite myelin degeneration in the sciatic nerve (fig. 9) followed by a comparable axon degeneration. Thirty % of these chicks had a mild, scattered myelin degeneration in the spinal cord. When supplements of riboflavin, pantothenic acid, and pyridoxine were given singly or collectively, there was no alleviation of the gross or pathological changes.

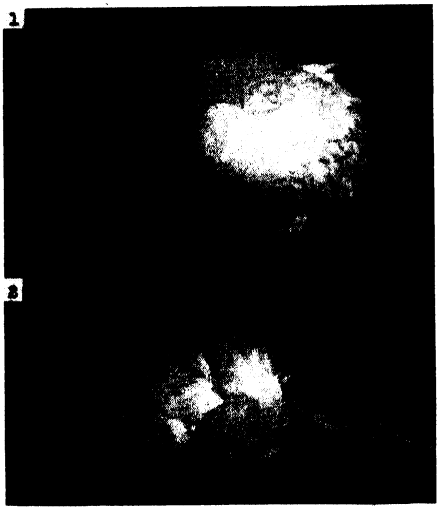

Fig. 1 A chick in an early stage of the paralysis observed in chronic thiamine deficiency.
Fig. 2 A chick in a much later stage of the same type of paralysis.

The chicks in group 9 which received a restricted amount of the ration equivalent to that eaten by chicks of the same weight in group 4, showed no signs of "curled toe" or extension of the legs. Instead they became very weak and flaccid with no sign of any rigidity of the leg even in the final stages of starvation. A rather unexpected observation was noted in connection with these starved chicks because they died slightly sooner than those with a chronic thiamine deficiency. There was a mild myelin degeneration in the sciatic nerves (fig. 7) and spinal cord of all the starved chicks. This degeneration seemed to be accompanied by some slight changes in the axon cylinders.

The chicks in group 10 which received the ration ad libitum with ample thiamine, grew normally and showed no signs of paralysis. Upon autopsy and histological examination, the spinal cord and the sciatic nerve (fig. 4) appeared to be normal in all respects.

The pyruvic acid concentration in the blood of six chronically thiamine deficient chicks averaged 6.3 mg. per 100 ml. in comparison with an average of 2.3 mg. per 100 ml. in six chicks which received ample thiamine. The pyruvic acid concentration was 0.17 mg. per gram in the sciatic nerve of the deficient chicks and 0.09 mg. per gram in the controls. There was little difference in the levels in the gastrocnemius muscle.

DISCUSSION

The differences in the outward symptoms and the pathological lesions which we observed in the acute and chronic phases of thiamine deficiency in the pigeon and the chick have been described above.

In pigeons with an acute thiamine deficiency but which received an ample amount of ration, a mild myelin degeneration was observed. However, in chicks with an uncomplicated, acute thiamine deficiency, no neuropathological lesions were observed. This difference may be accounted for by the much longer period necessary to produce an acute deficiency in the pigeon than in the young, growing chick. The complicating deficiencies that were present in the modified Swank pigeon ration may also be involved. The nutrition of the pigeon is not understood sufficiently well to permit one to evaluate the full extent of the inadequacy of this ration. However, it is significant that we found supplementing with the known crystalline B complex vitamins and sulfited 1 : 20 liver extract did not make the ration adequate.

It was observed that, in a prolonged thiamine deficiency in the pigeon and the chick, there was a definite myelin and axon degeneration of the peripheral nerves and in some cases, a similar degeneration in the spinal cord. The severity of the peripheral nerve lesions was greatest in the chicks in experiment 2 where the ration was complete in all known respects except thiamine. These observations were in accord with those of chronic thiamine deficiency made by Prickett, Salmon and Schrader ('39), Zimmerman ('39, '40), Street, Zimmerman, Cowgill, Hoff and Fox ('41), and Swank ('40).

Prolonged chronic thiamine deficiency leads to very definite complications because of the severe anorexia which thiamine deficient animals develop. Prickett, Salmon and Schrader ('39), and Street, Zimmerman, Cowgill, Hoff and Fox ('41) have reported a myelin degeneration in the peripheral nervous system of animals which had been maintained for long periods of time on the amount of food consumed by the thiamine deficient animals. These authors reported that the lesions observed in inanition were less severe than the ones observed in the prolonged thiamine deficient animals.

The work of Swank ('40); Swank and Bessey ('41), has not completely clarified the relation between the pathology of a prolonged thiamine deficiency and of inanition. He reported that in his forced-fed pigeons the lesions of the central nervous system observed in the chronically thiamine deficient birds were due to the thiamine deficiency and not to inanition. He believed that the daily introduction of a sufficient volume of ration into the crop of the pigeon, insured the digestion and utilization of the ration. However, our observations do not support this assumption. After the pigeons had been forced-fed for a period long enough to produce the initial symptoms of a mild chronic thiamine deficiency, the birds were observed to utilize only a small amount of the ration introduced daily into their crops. The pigeons were sufficiently accustomed to the forced feeding to endure this without vomiting. McCarrison ('19) postulated that a large part of the clinical and morbid anatomical effects observed in pigeons on an exclusive dietary of autoclaved rice could be attributed to a derangement of function of the organs of digestion and assimilation. This may explain the failure of the pigeon to utilize an adequate amount of food. The natural tendency of the bird to reduce its caloric intake during the thiamine deficiency could not be overcome by introducing food into the upper part of the digestive tract.

The role that inanition plays in the chronic thiamine deficiency cannot be too highly emphasized. Wolbach ('37), stated that a rational account of the pathological consequences of the deficiency of the B complex was not possible. He believed that it was better to regard the primary pathological effects of thiamine deficiency as not yet demonstrable and to regard the changes observed as secondary to the effects of inanition. Our observations support this view and these experiments with pigeons and chicks indicate that inanition is one of the principle factors involved. However, it should be pointed out that more severe degenerative changes occurred as the result of chronic thiamine deficiency than of inanition alone.

It is as yet impossible to explain why the control chicks which received the amount of ration voluntarily consumed by the chronically thiamine deficient chicks of the same weight died earlier than the deficient ones. The metabolism of the animal subjected to a prolonged period of partial thiamine deficiency is not understood. The role that inanition and other factors play in the production of neuropathological lesions during a chronic thiamine deficiency cannot be fully differentiated from the effects of the thiamine deficiency itself.

SUMMARY

1. There was a mild myelin degeneration in the peripheral nerves and in the spinal cord of the pigeons that had an acute thiamine deficiency. However, there was no evidence of any such degeneration in acute thiamine deficiency in chicks.

2. A mild myelin and axon degeneration was observed in the peripheral nerves and sometimes in the spinal cord of the pigeons and chicks which were maintained on a greatly restricted intake. These changes were more severe than those occurring in any acute deficiency produced in the pigeon.

3. Moderate degeneration occurred in the peripheral nerves and occasionally a mild degeneration in the spinal cord of the pigeons and chicks on a chronic thiamine deficiency. These changes were more severe than those occurring in inanition.

4. The pyruvic acid level of the blood of chronic thiamine deficient chicks was considerably higher than that of the controls. A slight increase in the pyruvic acid level of the sciatic nerve was detected but no change was observed in the gastrocnemius muscle.

These data do not warrant the conclusion that the neuropathology observed in a chronic thiamine deficiency can be attributed solely to a thiamine deficiency uncomplicated by other factors.

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PLATE 1

EXPLANATION OF FIGURES

3 A longitudinal section of the brachial nerve of a pigeon which received ample thiamine and the ration ad libitum. Normal myelination. Crossed Nicols. × 440.

4 A longitudinal section of the sciatic nerve of a chick which received ample thiamine and the ration ad libitum. Normal myelination. Marchi stain. × 440.

5 A longitudinal section of the sciatic nerve of a chick in a state of acute thiamine deficiency. Normal myelination. Crossed Nicols. × 440.

6 Another longitudinal section of the nerve in figure 5. Normal myelination. Marchi stain. × 440.

7 A longitudinal section of the sciatic nerve of a chick which had received the amount of ration consumed by a chronically thiamine deficient chick. Scattered myelin degeneration. Crossed Nicols. × 440.

8 A longitudinal section of the sciatic nerve of a pigeon in a state of chronic thiamine deficiency. Widespread myelin degeneration. Crossed Nicols. × 440.

9 A longitudinal section of the sciatic nerve of a chronically thiamine deficient chick. Widespread myelin degeneration. Marchi stain. × 440.

10 A longitudinal section of the sciatic nerve of a chronically thiamine deficient pigeon. Widespread axon degeneration. Bodian Ag stain. × 440.

11 A transverse section of the spinal cord of a chronically thiamine deficient pigeon. Scattered myelin degeneration. Marchi stain. × 440.

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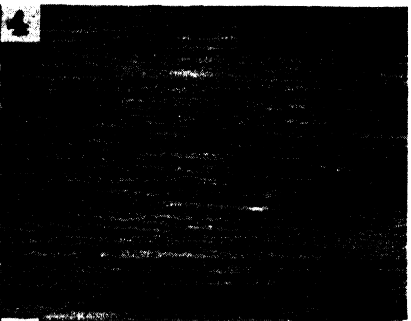
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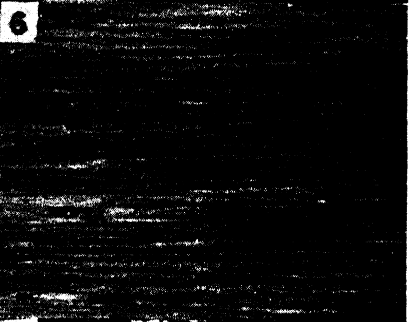
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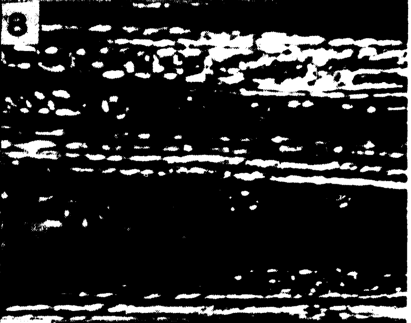
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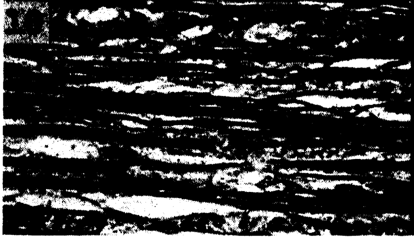
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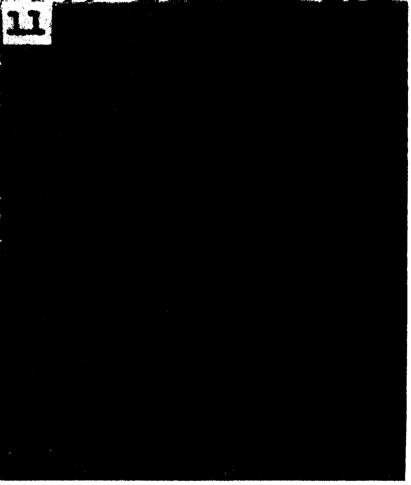
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EFFECTS ON THE ALBINO MOUSE OF FEEDING DIETS VERY DEFICIENT IN EACH OF SEVERAL VITAMIN B FACTORS

(THIAMINE, RIBOFLAVIN, PYRIDOXINE AND PANTOTHENIC ACID)[1]

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ONE FIGURE

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As part of the investigations in this laboratory, on the relation of diet to resistance to infection with the virus of poliomyelitis, the response of mice to single deficiencies of four members of the vitamin B group has been studied in some detail. In particular, was it necessary to know how the separate deficiencies would make themselves manifest and how long the animals would live after each deficiency became evident. The rat has been used extensively in studies on the several vitamins B, but less information is available concerning the manner in which an inadequate supply of these dietary essentials affects the mouse. It has, however, been amply demonstrated that there is a considerable difference in the response of these two species to deficiencies of some of the B factors.

Mice on a diet markedly deficient in vitamin B_1 become hyperirritable and develop anorexia and atonia, which are followed by death, but do not show several of the symptoms seen in the rat and considered to be specific for vitamin B_1 deficiency, such as loss of proprioceptive sense and a tendency to move in circles (Freudenberg and Cerecedo, '31; Hauschildt, '42; Woolley and White, '43). Likewise Foy and Cerecedo ('41) have reported that pyridoxine is essential for the mouse but in its absence there is no dermatitis such as that frequently observed in the rat on certain types of pyridoxine-deficient diets. On the other hand, the only consistent sign of a lack of riboflavin in the rat,

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[1] Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

besides a failure of growth, consists of changes in the eyes, and these may in some cases be discernible only upon special examination, whereas Lippincott and Morris ('41–'42) have described other riboflavin-deficiency symptoms in the mouse. The outstanding characteristics of these symptoms were loss of hair in certain areas, superficial cracks and fissures about the snout and on the front legs, exudate on the conjunctivae and about the mouth, a thickening and distortion of the ears, a hunched back while standing, and an abnormal gait with mild ataxia. Not all of these symptoms were observed in all of the animals, and a state of chronic deficiency produced more exaggerated symptoms than when the deficiency was acute.

Several investigators have studied pantothenic acid deficiency in the mouse (Morris and Lippincott, '41–'42; Sandza and Cerecedo, '41; Woolley, '41). The most commonly observed sign of pantothenic acid deficiency in the mouse appears to be alopecia, but Woolley has also reported hyperirritability, lack of muscular control followed by paralysis, and an abnormal condition of the eyes which, in some cases, could not be opened.

In the experiments reported below the vitamin B factors were supplied as pure compounds.[2] A deficiency of each of four factors, thiamine, riboflavin, pyridoxine, and pantothenic acid, was studied separately by omitting completely from the diet the factor under consideration. Results obtained in general confirm those reported in the literature but are more extensive, especially with respect to length of survival. They differ in certain other details.

EXPERIMENTAL

Two separate experiments were conducted, which, apart from some minor details, were similar except that the animals were raised on different stock diets. The mice used in the first experiment (experiment A) were raised on our regular stock diet which is partially composed of purified ingredients and has previously been described (Foster, Jones, Henle and Dorfman, '44). For the second experiment (experiment B) the animals were raised on a commercial dog food.[3] The two stock diets were used to determine if a difference in this respect would produce dissimilar results when the animals were put on the deficient diets. In each experiment the split-litter technique was used and the animals were distributed among five groups. There was considerable difference in the ages of the various animals, and at the time of starting, their ages varying from 28 to 41 days in both experiments, with the age of about 80% of the mice falling between the 32nd and 38th days.

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[2] These pure compounds were supplied by Merck and Company.

[3] Purina small dog checkers.

Group I was given a synthetic diet which was adequate for satisfactory growth; the composition of this diet is shown in table 1. The other groups of animals were given the same ration except that in each case one of the B factors was omitted, as follows: group II, thiamine; group III, riboflavin; group IV, pyridoxine, and group V, calcium pantothenate. In each experiment there were twenty animals in a group, with the exception of group I of experiment B, which contained nineteen animals. Experiment A was continued for 205 days, at which time all of the animals of groups II, III and IV, and all but four mice in group V, had died. Experiment B was continued for 135 days, at which time again all of the animals of groups II and IV were dead, and two and ten animals were still alive in groups III and V, respectively. The controls (group I) of experiment A were discontinued after 87 days, while those of experiment B were continued for the full 135-day period. The animals were weighed daily for the greater part of the experiment, after which they were weighed but once a week. In the second experiment daily food consumption of each animal was measured from the 12th to the 43rd day. The technique for measuring the food intake was the same as that previously described (Foster et al., '44). The weights of the animals in the corresponding groups in the two experiments were sufficiently similar at each weighing period to warrant combining them. The mice raised on the commercial dog food consisting of natural food materials were slightly heavier at the time the experiments were started

TABLE 1

Dietary ingredients.

BASAL DIET		VITAMIN B MIXTURE	
	Parts		*mg./100 gm.*
Labco casein	25.0	Thiamine chloride	0.2
Cellulose	2.0	Riboflavin	0.5
Salt mixture[1]	4.0	Pyridoxine	0.2
Linseed oil	1.5	Calcium pantothenate	5.0
Wheat germ oil	1.0	Nicotinic acid	10.0
Fish liver oil concentrate	0.008	Inositol	10.0
Cerelose	63.5	P-aminobenzoic acid	10.0
	97.008	Choline chloride	30.0
Vitamin B mixture	3.0	*The above was carried on:*	
	100.008	Cerelose	3.0 gm.

[1] Salts no. 12 (Jones and Foster, '42).

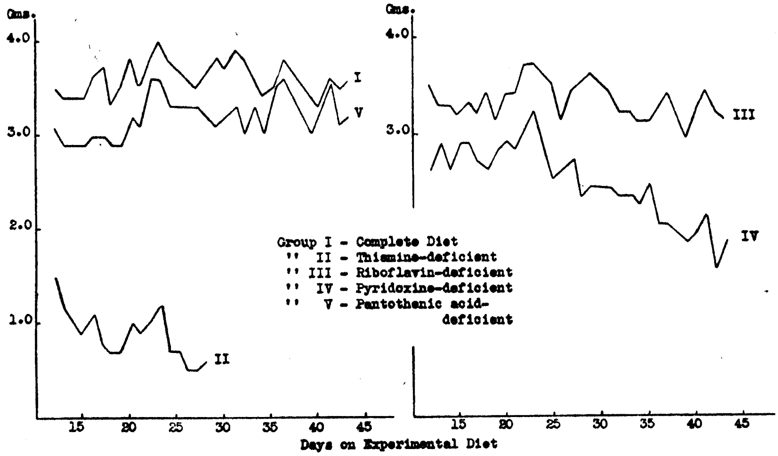
than were the mice raised on the stock diet which is partially composed of purified ingredients. This difference in weight was maintained throughout the experimental period in each of the groups except the control animals (group I). In this case the animals raised on the partially purified diet had attained a weight equal to that of the animals of the corresponding group raised on commercial dog food after approximately 1 month on the experimental diet.

RESULTS

The changes in weight of the animals for various intervals (usually 1 week) are presented in table 2, in which the combined averages of the weights of males and females of both experiments are given. Table 3 diagrammatically shows whether or not a significant difference in the weights of the animals of any two groups existed at the various weighing dates up to the 50th day. After this there was little or no change in this respect. The growth of the control animals on the complete diet (table 2, group I) was about equal to that of mice of the same age on a stock diet. All of the animals of this group appeared normal and none died. However, one was sacrificed for photographic purposes. In table 2 are also presented the numbers of survivors on each weighing day. In this case the males and females of each experiment are given separately.

Chart I

FOOD INTAKE



The food intake of the animals in experiment B is presented as curves (chart I). There was a definite and approximately uniform difference between the males and females of each group with respect to growth and food intake. For some unexplainable reason, there was a greater difference between the males and females in both the weights and food intake of the mice on the riboflavin-deficient diet than among any of the other groups. As expected, weights and intake of food of the females of all groups were somewhat less than those of the males.

Thiamine deficiency

The mice on the diet deficient in thiamine gained slightly in weight for a few days, but after about 10 days they lost weight rapidly. The first death occurred on the 19th day, after which the mortality rate was high (table 2, group II). The last animal died on the 31st day. As table 2 shows, there was only a slight difference in the survival times of the animals of experiments A and B. In addition to the marked loss of weight and subsequent death, the animals showed a marked anorexia and certain symptoms which may have been associated with the decreased food intake. Soon after the initial drop in weight the animals became hyperirritable, their backs were arched and their rear legs appeared straight and stiffened. A few days later these mice became generally hypotonic, with a gradually increasing tendency to drag their hind legs. Still later some of them lost their ability to stand and for the most part supported their weights on their bellies, with their legs spread out and rotated inward. At this stage some of them attempted to propel themselves by moving their heads on the floor of the cage. This was followed by a moribund state for a few hours and then death. It is to be noted that in agreement with other investigators, we did not observe in these mice, which were suffering from acute deficiency, the neuromuscular disturbances frequently seen in the rat. The diet used in this case was free of thiamine, but it should be stated that on diets in which the thiamine deficiency was less acute we have frequently observed the same symptoms as those characteristic of the rat. These symptoms may be described as follows: The early irritability and hypertonicity followed by gradual loss of muscular tone are similar to those described above, but before atonia appears there is a tendency for the deficient animal to tilt the head and trunk to one side and to move in circles. This is usually accompanied by a loss of proprioceptive sense and a tendency to fall over the edge of a table when allowed to move about freely. Ocasionally, shortly previous to death, there may be twitching movements of the legs and a retraction of the head or at times the head may be pulled forward.

TABLE 2

Weight data.

DAYS ON EXP. DIET	NUMBER OF SURVIVORS				AVERAGE WEIGHT (GRAMS)[1]
	Exp. A		Exp. B		
	♂	♀	♂	♀	
Group I — Complete					
2	11	9	8	11	14.3 ± .33
9	11	9	8	11	16.0 ± .26
16	11	9	8	11	17.6 ± .25
23	11	9	8	11	18.8 ± .25
30	11	9	8	11	19.9 ± .25
37	11	9	8	11	20.5 ± .25
44	11	9	8	11	20.6 ± .29
50	11	9	8	11	21.0 ± .24
69	11	9	8	11	21.9 ± .30
79	11	9	7	11	22.1 ± .31
Group II — Thiamine deficient					
2	10	10	9	11	14.5 ± .33
9	10	10	9	11	14.7 ± .31
16	10	10	9	10	11.3 ± .21
23	3	8	9	10	9.1 ± .25
30	0	1	1	3	
Group III — Riboflavin deficient					
2	11	9	8	12	15.1 ± .36
9	11	9	8	12	15.7 ± .31
16	11	9	8	12	16.2 ± .30
23	11	9	8	12	16.1 ± .33
30	11	9	8	12	17.0 ± .34
37	11	8	8	12	17.2 ± .42
44	9	8	8	12	17.1 ± .37
50	9	8	8	12	16.8 ± .42
69	5	7	8	11	17.3 ± .48
79	3	7	8	10	17.2 ± .59
98	1	7	7	8	17.0 ± .65
106	0	6	7	7	15.6 ± .49
113	–	6	5	7	16.4 ± .63
120	–	6	4	5	16.4 ± .70

DAYS ON EXP. DIET	NUMBER OF SURVIVORS				AVERAGE WEIGHT (GRAMS)[1]
	Exp. A		Exp. B		
	♂	♀	♂	♀	
Group IV — Pyridoxine deficient					
2	10	10	9	11	14.1 ± .36
9	10	10	9	11	15.6 ± .27
16	10	10	9	11	16.3 ± .28
23	10	10	9	11	16.0 ± .35
30	9	10	8	11	15.9 ± .34
37	7	10	8	10	14.7 ± .47
44	5	7	6	9	13.8 ± .50
50	3	5	6	7	12.5 ± .49
Group V — Pantothenic acid deficient					
2	10	10	9	11	15.1 ± .30
9	10	10	9	11	15.1 ± .22
16	10	10	9	11	16.3 ± .27
23	10	10	9	11	16.4 ± .31
30	10	10	9	11	17.5 ± .31
37	10	10	9	11	17.2 ± .33
44	10	10	9	11	17.6 ± .28
50	10	10	9	11	18.2 ± .33
69	8	10	9	11	18.4 ± .43
79	6	10	8	10	18.5 ± .46
98	5	10	7	9	18.1 ± .40
106	5	10	6	9	17.2 ± .41
113	5	8	4	8	18.3 ± .49
120	5	7	3	8	17.8 ± .52

[1] The weights are given for days after the start of experiment on which there were weight data available in both experiments on significant numbers of animals.

Average weight = the average of the four averages, or the average for animals on a given diet at a given time of the means for each sex for each experiment.

Standard error of the means, which follows the average weight, is computed from the pooled standard deviation measured within each sex and each experiment as follows:

$$\text{A. Pooled } \overline{\mathrm{SD}}^2 = \frac{\Sigma d^2_{Am} + \Sigma d^2_{Af} + \Sigma d^2_{Bm} + \Sigma d^2_{Bf}}{N_{Am} + N_{Af} + N_{Bm} + N_{Bf} - 4}$$

$$\text{B. SE of average of 4 averages} = \tfrac{1}{4}\sqrt{\left(\frac{\overline{\mathrm{SD}}^2}{N_{Am}} + \frac{\overline{\mathrm{SD}}^2}{N_{Af}} + \frac{\overline{\mathrm{SD}}^2}{N_{Bm}} + \frac{\overline{\mathrm{SD}}^2}{N_{Bf}}\right)}$$

The males of group III of experiment A dropped to 1 on the 98th day, and to 0 on the 106th day; therefore only the females of experiment A were included from this point on. Group V was the only other group carried beyond 98 days. There was no significant difference between the same three units (females of experiment A and males and females of experiment B) in group III and group V, out to the 120th day.

Riboflavin deficiency

The mice on the riboflavin-deficient diet continued to appear normal for about 3 weeks. They not only did not lose weight but actually gained weight at a rate only slightly below that of the mice on the complete diet. The first animal died on the 34th day and thereafter the mortality rate was low (table 2, group III). In the first experiment the last animal died on the 202nd day, and when the second experiment was discontinued on the 135th day, two were still alive. On the average the animals of experiment A died sooner than the animals of experiment B (table 2, group III). This difference appears too large to be the result of mere chance but until repeated, cannot be considered significant. The partially purified stock diet used in experiment A was well supplied with riboflavin as it contained 20% dried grass and 6% yeast.

TABLE 3

Diagrammatic presentation of the significance of the difference in weights among the various groups.[1]

DAY	HEAVY		LIGHT
9	I	III IV	II
		V	
16	I	IV V III	II
23	I	V III IV	II
30	I	V III	IV
37	I	V III	IV
44	I	V III	IV
50	I	V III	IV

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[1] The groups are represented in the order of their weight from heavy to light — left to right. Those in the same block are not significantly different from each other. At the 9th day group V was not significantly different from any of the others.

Of the forty mice on the riboflavin-deficient diet, twenty-three died without showing any specific symptoms. All of the others developed a rather characteristic skin lesion. These lesions made their appearance from the 44th to the 139th day, and although the numbers of animals showing them were about the same in the two experiments, they made their appearance earlier in experiment A (average 58 days) than in experiment B (average 96 days). They were definitely circumscribed, circular areas varying from a pin-point to 1 mm. in diameter and covered with a blood-red, moderately dense scab. The affected area contained from one to a dozen of these scabs. They appeared near the eyes, mouth, top of head or on the tail or ears. Usually, only one or two areas were affected in an individual animal. The ears became most severely damaged. The lesions spread over the surfaces of the ear and the areas became eroded and then necrotic. In some cases the entire ear sloughed off. The lesions seen in this laboratory are similar to but not identical with those described by Lippincott and Morris ('41–'42).

Day, Darby and Langston ('37) have reported cataracts in rats on a diet deficient in riboflavin. Cataracts were not observed in our mice, but no slit lamp or other special examination was made.

Pyridoxine deficiency

The mice on the pyridoxine-deficient diet grew slowly for 2 or 3 weeks, after which there was a rather marked decline in weight followed by death. The first animal died on the 28th day and the last one on the 67th day. There was practically no difference in survival time of the animals raised on the different stock diets (table 2, group IV). In agreement with Foy and Cerecedo ('41) no specific symptoms were observed. The general decline which preceded death was rather gradual; in some cases the animals lived as long as 41 days after the first signs of general debility were noted. During this period of deficiency, nearly all of the mice excreted a characteristic pigment in the urine, which was noted on the filter papers covering the floor of the cages. No study has been made of this pigment but it stained the paper a brownish-yellow with a slightly reddish tint. The staining was more pronounced and definitely different from that produced by normal urine. Lepkovsky and Nielsen ('42) have described a pigment obtained in the urine of rats on a diet deficient in pyridoxine.

Pantothenic acid deficiency

The mice on the diet deficient in pantothenic acid grew very little but on the other hand, there was very little loss of weight, even during the period of marked symptoms and occasional deaths. The first death occurred on the 67th day. In the first experiment there were four survivors at the end of the experiment (205 days) and in the second experiment (135 days) there were ten survivors. Again there was no real difference in this respect between the animals of experiment A and experiment B (table 2, group V). The symptoms seen were very similar to those described by Woolley ('41) with the exception that the only neuromuscular symptoms observed were profound spasticity of the extremities, acute arching of the spine, and an awkward gait. At no time were tremors, convulsions or paralysis noted. All of the animals showed some loss of hair, varying in degree from a mere thinning of the fur to complete loss except on the head. There was also a marked tendency toward dryness of the skin with the development of scaly desquamation. A few cases presented areas 5 to 10 mm. in diameter where the skin appeared to have been scratched off. Sixteen of the 40 mice showed hyperemia and edema of the eyelids.

SUMMARY

Using a diet in which the B factors were supplied as pure compounds, a deficiency of each of four of the factors (thiamine, riboflavin, pyridoxine and pantothenic acid) in the albino mouse has been studied.

When thiamine was omitted from the diet the animals lived from 19 to 31 days. They did not show symptoms characteristic of this deficiency as are seen in the rat. In less acute vitamin B_1 deficiency the classical polyneuritic symptoms have been observed in this laboratory.

Mice on the riboflavin-deficient diet lived from 34 to 202 days. Many of them developed characteristic dermatitis about the head and especially on the ears. In some cases nearly the entire ear sloughed off.

No specific symptoms or lesions (other than poor growth and death) resulted from the omission of pyridoxine from the diet. The animals lived from 28 to 67 days. Nearly all of the mice on this diet excreted in the urine a characteristic brownish-yellow pigment.

The first death on the diet lacking in pantothenic acid occurred on the 67th day and there was a survival of 21% when the experiment was discontinued (205 days). A loss of hair was the most outstanding sign of deficiency in these animals. In addition, a number of the mice developed some of the following signs: a profound spasticity of the extremities, acute arching of the spine, awkward gait, dryness of the skin with scaly desquamation, and hyperemia and edema of the eyelids.

ACKNOWLEDGMENT

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THE NICOTINIC ACID, PANTOTHENIC ACID, CHOLINE AND BIOTIN CONTENT OF FRESH, IRRADIATED EVAPORATED AND DRY MILK

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A number of values for the nicotinic acid, pantothenic acid, choline and biotin content of fresh and processed milk appear scattered through the vitamin literature. Many of the studies, however, contain too few values to permit the estimation of the variability which may occur between different samples of the same type of milk. Moreover, there are only a few studies in which values for fresh and processed milks have been obtained by the same methods and by the same investigators. The present study was undertaken to give us further information on the nicotinic acid, pantothenic acid, choline and biotin content of milk, to outline the variability that might be expected in the content of these factors in milk and especially to determine whether any significant losses occur when evaporated and dry milk are manufactured from fresh milk.

While pertinent values supporting or disagreeing with the data reported in this paper will be mentioned from time to time, it is not the purpose of the paper to include a comprehensive review of the subject under investigation.

The microbiological methods used in this investigation while highly useful are still subject to further refinements and perhaps to eventual displacement. The data obtained by the use of these methods are presented in the belief that they are the best obtainable at present.

NICOTINIC ACID

A more thorough investigation of the nicotinic acid content of milk has been made since the place of this vitamin in human nutrition is better understood than that of the other three vitamins and because the method used for the assays appears to be one of the better microbiological methods. In our laboratory the method of Snell and Wright ('41) as modified by Krehl, Strong and Elvehjem ('43) has given excellent results as judged by the reproducibility of the data, a minimum of tendency to drift and agreement with results secured by other laboratories.

The results of our microbiological assays for nicotinic acid in milk products are presented in table 1. The fresh milk samples were from Illinois. The evaporated milk samples were from more than twenty different plants well distributed geographically from Maryland to California, and from Mississippi to Wisconsin. The dry milk samples were from Wisconsin, Michigan and Utah. The processed milks were all analyzed within a few weeks after manufacture. The samples were taken during May. The differences found between the various types of milk are not believed to be significant. There is no indication that the processing of either irradiated evaporated milk or dry milk causes any loss of nicotinic acid. Since the heat treatment employed in the preparation of evaporated milk or dry milk is usually more severe than pasteurization, there is little possibility of nicotinic acid destruction by the latter process.

TABLE 1

Nicotinic acid, pantothenic acid, choline and biotin content of fresh, irradiated evaporated and dry milk on a fresh or reconstituted basis.[1]

TYPE OF MILK	NICOTINIC ACID			PANTOTHENIC ACID			CHOLINE			BIOTIN		
	No. of samples	Milligrams per liter		No. of samples	Milligrams per liter		No. of samples	Milligrams per liter		No. of samples	Micrograms per liter	
		avg.	range		avg.	range		avg.	range		avg.	range
Fresh	31	0.91	0.74–1.14	23	3.1	1.9–4.2	10	149	131–169	10	47	32–84
Irradiated evaporated	44	0.98	0.83–1.28	27	3.1	2.4–4.1	6	131	90–208	10	45	29–56
Dry skim	24	0.87	0.72–1.18	16	3.6	3.1–4.3	9	104	54–160	9	34	28–40
Dry whole	4	0.87	0.79–0.91	2	3.3	3.2–3.5	9	142	83–229	9	47	29–58

[1] By multiplying the reconstituted values for evaporated, dry whole, and dry skim milk by 2, 7.7, and 10.3, respectively, they may be converted to an undiluted basis.

The results presented in table 1 for nicotinic acid agree very well with values found by other investigators. The average value for fresh milk is 0.91 mg. per liter. Snell and Wright ('41) found 0.84 µg. of nicotinic acid per milliliter of milk and Teply, Strong and Elvehjem ('42) found 0.08 mg. per cent for whole milk, 0.18 for evaporated milk and 0.89 for skim milk powder on an undiluted basis. Noll and Jansen ('41) using a chemical method found 0.6–0.9 µg. of nicotinic acid per milliliter of skim milk.

PANTOTHENIC ACID

The samples used for pantothenic acid assays were obtained during June and July. All processed samples were assayed soon after preparation. The fresh milk samples were from Illinois; evaporated milk samples were from Ohio, Kentucky, Wisconsin, Illinois, Tennessee and Maryland; samples of dry milk were from Wisconsin, Michigan and Utah.

The method used for pantothenic acid assays was that proposed by Hoag, Sarett and Cheldelin ('44). This method uses Lactobacillus arabinosus rather than Lactobacillus casei as the test organism. A more rapid and greater total growth is secured with L. arabinosus and in addition in certain instances the response is perhaps more specific for pantothenic acid than with L. casei. Since the results reported in this paper were obtained without enzymatic treatment or special hydrolysis they represent largely if not entirely free pantothenic acid. However, Atkin, Williams, Schultz, and Frey ('44) report that milk does not contain bound pantothenic acid and data for free pantothenic acid in this product may be fairly indicative of the total content of the vitamin.

Assays were made directly on diluted fresh and dry skim milk. For evaporated milk and dry whole milk a 5% metaphosphoric acid filtrate was prepared. After the protein and fat were separated the filtrate was neutralized before addition to the assay tubes. Direct assays of evaporated, dry whole milk and other milk which had been homogenized consistently gave erratic results which were often 50% or more higher than those obtained with fresh milk. Investigations which cannot be reported in detail here show that the interference which is apparently caused by homogenized milk fat can be eliminated by preparation of the metaphosphoric acid filtrate.

The data on the pantothenic acid content of fresh and processed milk are included in table 1. No significant differences are shown. The range of results indicates a considerable variation in the pantothenic acid content of milk. The average value found for fresh milk, 3.1 µg./ml., compares favorably with the values of 3.2 and 3.3 found by Atkins, Williams, Schultz and Frey ('44). The value of 4.0 µg./ml. found by Strong, Feeney and Earle ('41) is within the range of values reported in table 1.

CHOLINE

The sources of samples for the choline investigation were similar to those for the pantothenic acid assays except that the evaporated milk samples were drawn from a wider range. All the samples were collected during February and March.

The method employed was essentially that of Horowitz and Beadle ('43) using the "cholineless" mutant no. 34486 of Neurospora crassa. The method was slightly modified by the use of 10 ml. of medium and 50 ml. flasks rather than 25 ml. of medium and 250 ml. flasks and by the omission of the permutit exchange. The latter step is included in the original procedure specifically to eliminate methionine, but also probably excludes some other interfering substances. In separate experiments, which will not be reported here, and by calculation, it has been found that the amount of methionine or other interfering substances present in milk is not sufficient to produce significant errors in the assay if the data are taken from the lower portion of the assay curve. If this precaution is observed most of the values are accurate within 10% and probably all are within 20%. Recoveries of choline added to fresh milk ranged from 85 to 102% and averaged 96%.

The average value reported in table 1 for fresh milk, 149 mg. per liter, is in excellent agreement with that found with a chemical method by Engle ('43), namely, 147 mg. per kilogram. The data in table 1 suggest that dry skim milk contains less choline than fresh milk. An explanation for this is the loss of some choline in the phospholipid fraction of the cream.

BIOTIN

For the biotin assays the "cholineless" mutant of Neurospora crassa was used. This organism requires only the two vitamins, choline and biotin, and is well suited for biotin estimations. The wild type of Neurospora crassa should be equally useful but this necessitates the maintenance of a separate stock. One must acknowledge that the specificity for biotin of neurospora and of other test organisms is still open to question until further investigations have been made of compounds which supplant biotin or which antagonize the organism's response to biotin. The wild type of Neurospora crassa responds to desthiobiotin as has been shown by Lilly and Leonian ('44). If this compound should be present in milk, the values in table 1 would reflect its presence as additional biotin.

The sources of the samples used for the biotin investigation were similar to those for choline. The samples were obtained during February, March and April.

The figures for biotin presented in table 1 are somewhat misleading. The presence of a few high values in the fresh, irradiated evaporated and dry whole milk series widened the range of the results and markedly raised the average. Most of the values fell between 30 and 40 μg. per liter and thus were in good agreement with the 30–40 mμg. per milliliter (30–40 μg. per liter) reported by Lampen, Bahler, and Peterson ('42), who used Cl. butylicum as the test organism. No high values were encountered in the dry skim milk series and this is reflected in the lower average.

SUMMARY

1. The average nicotinic acid, pantothenic acid, choline and biotin content of fresh milk was found to be 0.91 mg., 3.1 mg., 149 mg. and 47.1 μg. per liter, respectively.

2. In the processing of irradiated evaporated, dry skim or dry whole milk, the data show no significant losses in the contents of nicotinic acid, pantothenic acid, choline and biotin with the possible exception of the slightly lower biotin content of dry skim milk.

3. The possibility of small and probably insignificant processing losses cannot be definitely excluded because the raw milk sources of the different types of milk tested were not identical, because of the variability of the data, and because of the limitations in accuracy of the microbiological methods used.

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THE EFFECT OF SUCCINYLSULFATHIAZOLE AND PHTHALYLSULFATHIAZOLE ON THE BACTERIAL FLORA OF RAT FECES

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The feeding of sulfonamides that are poorly absorbed from the intestinal tract [e.g., sulfaguanidine, succinylsulfathiazole (SST) and phthalylsulfathiazole (PST)] causes a decrease in the coliform count in the feces of the dog (Poth et al., '42), rat (Gant et al., '43), monkey (Welch et al., '42), mouse (White, '42) and man (Marshall et al., '40). Despite this decrease, the total number of organisms in such feces is reported to remain relatively constant (Light et al., '42), or actually to increase (Gant et al., '43). The prolonged feeding of such sulfonamides in highly purified diets results, in the rat, in the development of signs of deficiencies (Welch and Wright, '43; Black et al., '42) that are not seen when the purified diets are fed without these sulfonamides, or when the drugs are added to ordinary commercial rations.

It has been suggested (Black et al., '41) that these signs of deficiency are caused by the inhibitory action of the drug on vitamin-synthesizing bacteria in the intestine, and that Escherichia coli, in particular is the organism so affected. In this connection Gant et al. ('43) suggested that, even though the coliform count of rats continued on the drug-diet returns to normal, such organisms represent sulfonamide-resistant strains that have lost the ability to synthesize the growth factors involved.

In view of these reports it seemed advisable to study the influence of diet on the fecal flora of rats, and to investigate the vitamin-synthesizing powers of a sulfonamide-resistant organism.

EXPERIMENT I

Techniques. A fecal pellet for bacteriological examination was gently expressed from the rectum of the rat, and was allowed to fall into a sterile petri dish. Using aseptic techniques, the pellet was transferred to a graduated centrifuge tube and emulsified in saline solution with a swab stick. After dilutions of the original suspension were made, the tube of emulsified feces was centrifuged for 20 minutes at 1700 revolutions per minute, and the approximate volume of solid material was measured. It was assumed that 1 ml. of solid was equivalent to 1 gm. (wet weight) of feces, and on this basis the dilutions of the fecal material were calculated.

Suitable amounts were transferred routinely from the dilution tubes to Durham tubes of bromcresol purple-lactose broth and to tubes of freshly steamed thioglycollate medium. Occasionally plates were made with nutrient agar or desoxycholate agar. After these inoculations were made, the dilution tubes sometimes were held at 80°C. for 10 minutes to destroy the vegetative forms of bacteria. A second set of thioglycollate tubes then was inoculated. Tubes and plates were incubated at 37°C., and were read after 72 hours. All media used contained p-aminobenzoic acid (0.001%).

The number of aerobic organisms present in a sample was calculated from the lactose tube of greatest dilution that showed growth. The production of acid and gas in the lactose tubes was taken as presumptive evidence of the presence of coliform organisms. As confirming evidence, streaks on desoxycholate plates were made from the two highest dilution tubes showing such acid and gas, and the plates were examined after 24 hours for the presence of typical coliform colonies. Growth in the bottoms of the thioglycollate tubes was used as a measure of anaerobic organisms; similar growth in the second set of thioglycollate tubes (those inoculated from the heated dilutions) was used as a measure of the presence of anaerobic spores in the original material.

Effects of succinylsulfathiazole. Six 50-day-old piebald rats from the Sunny Hill strain were placed in individual cages with wide-meshed screen floors and fed stock ration [1] and water ad libitum. Table 1 includes the average total counts of "aerobes" (including coliform bacteria), "anaerobes," and coliforms in the feces of these rats. It should be mentioned that the figures in this table average not only the variation which occurred among the six rats, but also include the normal fluctuation always observed from count to count for any one rat. Counts on one of these rats were continued for 146 days, during which time the average number of aerobic organisms was 10^8; anaerobes, 10^7; coliforms, 10^5; and anaerobic spores, 10^5 per gram of feces.

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[1] Purina chow.

After 21 days on the stock ration, five of the rats described above were placed on a highly purified diet (table 2). Within a day the fecal pellets changed from the more bulky, brown, and softer state characteristic of the animals given the stock ration to a blue-green, compact mass. Almost all the fecal samples showed a drop in each of the three counts on the first or second day; this was followed by a gradual return to higher levels. Table 1 shows the results of these counts over a period of 15 days.

TABLE 1

Average counts of bacterial flora of rat feces. Effect of age and diet on the rat.

DIET	AEROBIC ORGANISMS		ANAEROBIC ORGANISMS		COLIFORM ORGANISMS		ANAEROBIC SPORES	
	Exp. I[1]	Exp. II[2]	Exp. I	Exp. II	Exp. I	Exp. II	Exp. I	Exp. II
Succinylsulfathiazole tests								
Stock	10^{9}(35)	10^{9}(9)	10^{8}(25)	10^{8}(8)	10^{7}(34)	10^{8}(8)	10^{5}(3)	10^{4}(8)
Purified	10^{8}(19)	10^{8}(11)	10^{7}(21)	10^{8}(6)	10^{6}(23)	10^{6}(11)	10^{4}(4)	10^{2}(6)
0.5% SST		10^{8}(8)		10^{8}(6)		$<10^{2}$(5) [3,4]		10^{3}(6)
2% SST	10^{8}(39)	10^{7}(6)	10^{9}(39)	10^{7}(4)	10^{3}(41)	$<10^{2}$(4) [4]	10^{2}(10)	10^{3}(4)
Phthalylsulfathiazole tests								
Purified	10^{8}(17)		10^{8}(17)		10^{7}(17)		10^{5}(2)	
0.5% PST	10^{8}(21)	10^{7}(4)	10^{8}(21)	10^{7}(3)	10^{1}(21)	$<10^{1}$(3) [4]	10^{3}(5)	10^{3}(3)
1% PST	10^{8}(19)		10^{9}(19)		$<10^{1}$(19)		10^{3}(3)	
2% PST	10^{8}(20)	10^{8}(6)	10^{9}(20)	10^{8}(4)	$<10^{1}$(20)	$<10^{2}$(6)	10^{3}(5)	10^{3}(4)

Counts are given as per gram of feces (wet weight).

Figures in parentheses indicate the number of counts included in the average.

[1] In experiment I the rats were about 75 days old when placed on the succinylsulfathiazole diets, while the rats given phthalysulfathiazole were about 40 days old when placed on the drug diet.

[2] In experiment II all the rats were about 60 days old when placed on drug diets.

[3] The counts for one rat in this group remained above this average.

[4] After 3 days on the drug diet.

Two of these rats were continued on this purified diet for 132 days. Average fecal counts of these animals during this period were: aerobes, 10^8; anaerobes, 10^7; coliforms, 10^6; and anaerobic spores, 10^4 per gram of feces.

At the end of the above 15-day period three of the rats on the purified ration were placed on a diet in which 2% SST replaced an equal amount of sucrose. The slight diarrhea which Gant et al. ('43) observed when SST was added to a purified diet has not been seen in this laboratory, probably because "Cellu flour" was incorporated in the basal diet. Although no change in the physical characteristics of the fecal pellets occurred, there was an immediate drop in the coliform count. Following this initial drop, considerable variation was noted in the coliform counts of these rats: for one animal such variation was quite small and the average value was 10^5 per gram of feces; counts on another animal fluctuated widely ($0–10^6$) and averaged 10^3 coliform organisms per gram; feces from the third rat were almost entirely devoid of coliform organisms, after the addition of the drug to the diet, until near the end of the experiment, when the count gradually rose to 10^4 per gram. Counts over a period of 119 days are given in table 1.

TABLE 2

Composition of the purified diet.

INGREDIENTS	PARTS	CONTENT OF VITAMIN MIXTURES	*gm.*
Sucrose	61.78	Vitamin A D E concentrate (450,000 U.S.P. units A and 90,000 U.S.P. units D per g.)	7
Casein (vitamin free)	18.00	α-Tocopherol	2
Primex vegetable oil	10.00	Corn oil	41
Salt mixture (Hubbell, Mendel and Wakeman, '37)	4.00	Vitamin mixture:	
Cellu flour	4.00	Inositol	2.0
Corn oil	2.00	Ca pantothenate	1.1
Choline chloride	0.10	p-Aminobenzoic acid	1.0
Vitamin A D E concentrate	0.10	Nicotinic acid	1.0
Vitamin mixture	0.0236	Riboflavin	0.4
Vitamin K (2-methyl 1-4 naphthohydroquinone diacetate)	0.0010	Pyridoxine HCl	0.2
Total ingredients	100.0046	Thiamine HCl	0.2

Reference to table 1 shows that the differences in the counts of the total aerobic and anerobic organisms, and of the anaerobic spores found in the feces of rats on these three diets, is at most a hundred fold and therefore is probably insignificant. However, the drop in the number of coliform organisms found in the feces of the rats changed to the diet containing 2% SST is significant.

Since the rats used in this experiment were relatively old when they were placed on the drug diet, deficiency symptoms characteristic of a 2% SST diet (Welch and Wright, '43) were slow in developing. The first noticeable symptom was a tendency for the eyelids to stick together; this was observed for one of the rats on the twentieth day of the diet. Later, spectacled eye, thinning of the hair on the head, loss of weight, slight graying of the hair, sore mouth, and porphyrin-caked whiskers were noted.

Starting on the 105th day of the drug diet, two of the three rats receiving this ration were given daily subcutaneous injections of a supplement containing folic acid concentrate[2] equivalent to 25 µg. of potency 40,000 material (Mitchell and Snell, '41) and 5 µg. of crystalline biotin. The drug diet and injections were continued for a period of 14 days. During this time the two supplemented rats improved in physical appearance (condition of eyes, whiskers, and hair) and were relatively constant in weight. Because these rats were essentially full grown, a marked increase in weight could not be expected. In the case of the unsupplemented rat receiving the drug diet, the deficiency symptoms became increasingly severe.

Fecal samples were collected and examined during this time. No appreciable changes were observed in the counts. This indicated that the curative effects of folic acid and biotin were brought about by replacement therapy and not by increasing the number of coliform organisms in the feces.

Immediately before and at the end of the period of supplemental feeding total leucocyte counts were made on blood from each of the three rats on the drug diet. Before supplementation all three rats had low counts (4,600–5,580). At the end of the experiment, the blood of the two supplemented rats showed normal counts (13,300; 17,100), while the count of the unsupplemented rat was still low (6,830). This confirms the work of Daft and Sebrell ('43), who reported the successful treatment with folic acid of the leukopenia which had developed in rats fed SST in a purified diet.

Twenty-four-hour fecal samples were collected from rats on each of the three diets described above. The pellets from a single rat were ground in a mortar and divided into two approximately equal samples, each of which was weighed and placed in a small pyrex bottle. One of the two samples was covered with 10 ml. of 6N sulfuric acid and then autoclaved at 15 pounds pressure for 1 hour. After cooling, the autoclaved sample was neutralized, diluted, and filtered. Suitable dilutions were made of the filtrate, and these were assayed for biotin by the microbiological method of Landy and Dicken ('42).

To the second sample of ground feces were added takadiastase (2% of the weight of the feces), 20 ml. of water, and 2 ml. of benzene. The bottle was tightly stoppered, and the mixture was allowed to digest at 37°C. for 24 hours, after which it was autoclaved for 15 minutes at 15 pounds pressure. After dilution, the sample was filtered, and the filtrate was assayed microbiologically for folic acid and pantothenic acid according to the method of Landy and Dicken ('42). The folic acid standard used in these tests was a concentrate containing 200 µg. of folic acid per gram.[3]

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[2] This concentrate was prepared from grass juice powder by Dr. L. D. Wright of these laboratories. The procedure used was similar to that described by Hutchings, Bohonos and Peterson ('41), and was carried through the superfiltrol eluate stage.

The results of these assays are shown in table 3. In almost all cases the feces from rats on the drug diet contained appreciably smaller amounts of biotin and folic acid than were found in the feces of rats on the stock and on the purified rations. Comparison of figures for the rats on the stock and the purified diets showed that, except for pantothenic acid, the greater daily elimination of the vitamins by the rats fed the stock diet was caused only by the larger quantity of feces excreted on this diet; the folic acid and biotin contents per gram of feces remained essentially unchanged.

TABLE 3

The effect of diet on the fecal excretion of biotin, folic acid, and pantothenic acid by the rat.

DIET	BIOTIN		FOLIC ACID		PANTOTHENIC ACID	
	µg./gm.	*µg./day*	*µg./gm.*	*µg./day*	*µg./gm.*	*µg./day*
Stock	0.91(5)[1]	8.6 (5)	5.5 (7)	42.7(7)	62 (6)	428 (6)
Purified	0.60(6)	0.78(6)	4.7 (9)	5.3 (9)	29.8(9)	35.4(9)
0.5% PST	0.09(4)	0.15(4)	0.36(5)	0.54(5)	17.9(5)	26 (5)
1% PST	0.08(3)	0.08(3)	0.59(4)	0.65(4)	12 (4)	10 (4)
2% PST	0.07(3)	0.13(3)	0.9 (4)	1.2 (4)	17 (4)	18 (4)
2% SST	0.28(4)	0.39(4)	2.5 (6)	4.2 (6)	16.9(6)	27.2(6)

[1] Figures in parentheses indicate the number of assays included in the average.

In this connection it is of interest to comment on the relative vitamin contents of the diets used. According to Taylor et al. ('42b) Purina Chow has the following approximate composition: pantothenic acid, 14 µg./gm.; biotin, 0.19 µg./gm.; and folic acid, 0.71 µg. of potency 40,000 material per gram. The purified diet, which was also the base for the drug diet, contained neither biotin nor folic acid except for possible traces associated with purified materials, but did contain 44 µg. of pantothenic acid per gram of diet. In spite of this greater pantothenic acid content, much less of this vitamin was excreted per gram of feces by the rats on the purified diet than by the rats on the stock ration.

Effects of phthalylsulfathiazole. A number of 38-day-old rats of the Rockland strain were divided into four groups. Group I received the purified diet; group II, the purified diet containing 0.5% PST; group III, the purified diet containing 1% PST; and group IV, the purified diet containing 2% PST. The rats in group IV did not show normal weight gains, nor was their physical appearance good. Several from this group died before the end of the experiment.

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[3] Kindly provided by the Lederle Laboratories.

The outstanding effect of the addition of PST to the diets was a definite decrease in the number of coliform organisms present in a given weight of feces. This is shown in table 1 in which counts taken over a period of 77 days are shown. From the rats fed the two higher drug levels the coliform counts became very low within the first week after the addition of the drug to the diet, and remained low throughout the entire experiment. Although a definite reduction in the number of coliform organisms occurred in the feces of rats fed diets containing 0.5% PST this reduction was not maintained consistently. The count varied from 0 to 10^4 coliforms per gram of feces.

Beginning on the fifty-sixth day of the experiment, a rat from group II (0.5% drug) and one from group IV (2% drug) were each given, by stomach tube, daily supplements of 5 µg. of crystalline biotin and folic acid concentrate equivalent to 25 µg. of potency 40,000 material. Both rats showed an immediate improvement in physical appearance and a marked gain in weight. The supplemented drug diet was fed for 3 weeks, during which time there was no appreciable change in the bacterial counts of the feces of either rat.

In addition to the bacterial counts made on the feces of rats fed these diets, occasional microbiological assays also were conducted to measure the amounts of biotin, pantothenic acid, and folic acid eliminated in the feces. Twenty-four-hour samples were collected and treated as outlined above for the rats given SST. Appreciably smaller amounts of biotin and folic acid were excreted by rats receiving the drug-containing diets. Table 3 shows average results obtained from these assays.

EXPERIMENT II

Data from experiment I showed that the fecal coliform counts of the young rats fed PST were reduced to a lower level than were the coliform counts of the older rats which were fed SST. A second experiment was set up using 50-day-old rats of the Albino Farms strain to determine whether the difference in the ages of the rats used in experiment I had any influence on the results obtained. Bacterial counts for experiment II are included in table 1. Here the 2% level of either drug reduced the fecal coliform counts to a low figure. This reduction, however, occurred more rapidly when PST was used than when an equivalent percentage of SST was included in the diet.

In this experiment the animals were maintained on the stock ration for 3 days after which time all but three of the rats were fed the purified diet for a period of 5 days. Both 0.5 and 2% levels of PST and of SST were then added to the purified diets of some of these rats. Supplementation of the diet with folic acid and biotin was not carried out in this experiment.

The bacteriological techniques used were as described for experiment I except that 0.5% dextrose-beef heart infusion broth tubes were inoculated and incubated in Weiss-Spaulding jars for the determination of the anaerobic and anaerobic spore counts.

EXPERIMENT III

While experiments I and II were in progress, a laboratory strain of Escherichia coli was made resistant to sulfonamides by daily transfers in bacto-peptone broth containing gradually increasing amounts of sulfanilamide (SA). When growth studies showed the strain to be resistant to M/100 SA (in bacto-peptone medium), a comparison was made of the amount of folic acid found per milliliter of culture of the resistant and of the parent strain. The folic acid standard used in these assays was a solution of crystalline folic acid.[4]

When the tests were run in bacto-peptone medium containing no sulfonamide, the parent strain developed, in 18–24 hours, a turbidity of 257–375 units, as measured by a Klett-Summerson photoelectric colorimeter, and assays by Streptococcus fecalis (the so-called Streptococcus lactis R strain) showed 0.018 to 0.026 μg. of folic acid per milliliter of culture. Under the same conditions, the resistant strain developed a turbidity of 214–260 units, with 0.0098–0.0196 μg. of folic acid per milliliter of culture.

When the test flasks contained M/100 SA in the medium, and large inocula were used (1 ml. per 20 ml. of medium, as was also used above), the parent strain developed a turbidity of 208–241 units after 18–24 hours growth, and 0–0.0004 μg. of folic acid was found per milliliter of culture. Under similar conditions, the resistant strain showed a turbidity of 300–400 units, and produced 0.0016–0.0051 μg. of folic acid per milliliter of culture. Adequate controls were run to correct for the amount of folic acid present in the uninoculated medium, and to demonstrate that the amount of sulfonamide used had no inhibitory effect on the assay organism.

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[4] Lederle Laboratories.

These results indicate that the presence of the sulfonamide in the medium, and not the state of sulfonamide resistance of the organism, determines the amount of folic acid produced. Details of the experiments quoted here, and of two tests using sulfathiazole as the sulfonamide, are given in table 4.

TABLE 4

Folic acid synthesis by a sulfonamide resistant and a sulfonamide sensitive strain of Escherichia coli.

TEST NO.	MEDIUM	HOURS INCUBATION	SENSITIVE STRAIN		RESISTANT STRAIN	
			Turbidity	Folic acid	Turbidity	Folic acid
				μg./ml. culture		*μg/ml. culture*
I	BP [1]	24	355	0.026	220	0.0098
II	BP	24	375	0.024	260	0.0128
III	BP	24	305	0.018	216	0.0093
IV	BP	18	257	0.020	315	0.0196
V	BP	18	289	0.019	214	0.0173
I	BPSA [2]	24	230	0.0002	350	0.0016
II	BPSA	24	226	0.0004	330	0.0051
IV	BPSA	18	208	0.0003	397	0.0051
V	BPSA	18	241	0.0000	400	0.0047
IV	BPST [3]	18	142	0.0002	180	0.0023
V	BPST	18	137	0.0000	213	0.0060

[1] BP = Bacto-peptone medium.

[2] BPSA = Bacto-peptone medium containing M/100 sulfanilamide.

[3] BPST = Bacto-peptone medium containing M/2000 sulfathiazole.

DISCUSSION

Bacteriological examination of the feces of rats showed that the number of organisms present varied from rat to rat and in the same animal from day to day. Changing the diet of the rat from Purina Chow to a highly purified diet containing adequate amounts of all the known growth factors did not change significantly the bacterial flora of the feces. However, when either SST or PST was added to such a purified diet, a noticeable drop in the number of coliform organisms in the feces occurred. No significant increase or decrease was observed in the number of aerobes present; presumably the decrease in the number of coliform organisms was balanced by an increase in other organisms.

In experiment I, when the drug included in the purified diet was SST (2%), the coliform counts varied over a rather wide range. Concentrations of PST at 1 or 2% of the diet proved much more effective in reducing the fecal coliform count. In this experiment, the rats receiving

PST were younger and of a strain differing from those receiving SST. In the experiment in which all the rats used were of the same strain and age, the 2% SST diet reduced the coliform count to as low a level as did the 2% PST diet, but 0.5% PST was more effective in reducing this count than was 0.5% SST.

Rats fed purified diets containing either drug developed, in time, symptoms usually associated with dietary deficiencies. The growth and weight gains were subnormal; thinning of the hair occurred on the head and, in some cases, on the abdomen; the coat became dull and dry; porphyrin-caked whiskers developed. In the longer experiment (I), especially in the rats fed SST, spectacled eye and slight graying of the hair were observed, and patches of black hair at the base of the fore-legs turned a deep chocolate brown in color. The supplemental feeding of biotin and folic acid resulted in a greatly improved physical appearance. Blood counts on the SST-fed rats showed a definite improvement in the total leucocyte count after such a period of vitamin feeding. The drug was not removed from the diet during the period of supplementation, and no change in the number, kind, or distribution of fecal organisms was noted.

Assays of 24-hour fecal samples from experiment I to determine the amounts of biotin, folic acid, and pantothenic acid excreted gave rather irregular results. Increasing amounts of biotin seemed to be excreted with the feces as the rat grew older. This is more clearly apparent in the older rats fed SST than in the younger rats fed PST, and may merely reflect the increase in the proportionate size of the cecum as the rats grew larger (Taylor et al., '42a). For folic acid and pantothenic acid, the limits of variation of vitamin per gram of feces, or of vitamin per day were rather wide, and this variation did not seem to have any relation to the age or diet of the rat. Animals fed the SST or PST diet excreted significantly lower amounts of biotin and folic acid than did those not receiving the drugs. Pantothenic acid levels in the feces of rats fed drug diets appear to be depressed.

Counts of the intestinal flora, as performed in these experiments, indicate that a more complete survey of the intestinal organisms might be of value in following the effects of these and similar drugs on fecal organisms, and perhaps would lead to a better understanding of the action of such compounds.

SUMMARY

When a highly purified diet containing 0.5% to 2% succinylsulfathiazole or phthalylsulfathiazole was fed to rats over a long period of time

there developed signs of nutritional deficiency which were corrected by the feeding of biotin and folic acid. The feeding of such sulfonamides also caused a decrease in the coliform count of the rat feces, but caused no significant change in the number of "total aerobes," "total anaerobes," or anaerobic spores in the feces. Lower levels of biotin, folic acid and pantothenic acid were excreted in the feces of rats fed diets containing the drugs than were excreted by rats fed the same diet without the drug.

Neither a sulfonamide-resistant nor a sulfonamide-sensitive strain of Escherichia coli synthesized as much folic acid when grown in the presence of sulfonamides as was synthesized during growth in a medium not containing the drug.

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EFFECTS OF VARIATIONS IN DIETARY VITAMIN C ON THE PHYSICAL WELL BEING OF MANUAL WORKERS[1]

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INTRODUCTION

The primary purpose of this study was to examine carefully the early effects of total deprivation of vitamin C on the efficiency of manual workers. Information was also sought on the questions whether 75 mg. of ascorbic acid daily suffice for such workers and whether a daily supplement of ascorbic acid added to a good diet has any measurable effects in 2 months.

Our observations centered around physical fitness because of the statements in the older literature that lethargy and inefficiency are the earliest symptoms of scurvy, far antedating the onset of clinical signs (see Pijoan and Lozner, '44). This was well shown in the study of Crandon, Lund and Dill ('40) on the course and end results of scurvy induced experimentally in one subject. He began to complain of fatigue in about 60 days, but did not show clinical scurvy until considerably later.

METHODS

The experiments were conducted during the summer in Civilian Public Service Camp 32, situated at Campton, New Hampshire, in the southern part of the White Mountains at an altitude of about 2,000 feet. The days were moderately warm and the nights cool.

The twenty-four volunteer subjects were engaged in a variety of jobs associated with the camp's work schedule, which included clerical work, kitchen work, camp maintenance, farming, and work in the woods on such duties as clearing trails and road building. The range of daily caloric expenditures, depending on the subject's job, was estimated to

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[1] This work was carried out under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the President and Fellows of Harvard College.

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be 2400 to 5000. The camp routine was disturbed as little as possible by the experiment, and the subjects carried on their usual work schedule throughout. Four subjects transferred to other projects for reasons unrelated to the experiment.

There were four groups of subjects (table 1). The "deficient group" was to demonstrate the effects of total deficiency; the "supplemented deficient group", the effects of 75 mg. of ascorbic acid daily; the "normal control group", the effects of a good normal diet; and the "supplemented normal control group", the effects of a normal diet supplemented with 75 mg. of ascorbic acid daily.

During 2 weeks of preliminary control (period 1) all groups ate the regular camp diet, which contained liberal amounts of fresh milk, eggs, meat, vegetables and fruit, provided 60 to 100 mg. of ascorbic acid daily, and was good when judged by the National Research Council standards.

During the following 8 weeks of modified diet (period 2) the two control groups continued on the usual camp diet. Each "supplemented

TABLE 1

Organization of experiment.

	DEFICIENT GROUP (8 men)		SUPPLEMENTED DEFICIENT GROUP (8 men)		NORMAL CONTROL GROUP (4 men)	SUPPLEMENTED NORMAL CONTROL GROUP (4 men)
Period 1 Preliminary control, 2 weeks	Normal diet		Normal diet		Normal diet	Normal diet
Period 2 Modified diet, 8 weeks	Deficient diet + placeboes		Deficient diet + 75 mg. ascorbic acid daily		Normal diet + placeboes	Normal diet + 75 mg. ascorbic acid daily
Period 3 Final control, 2 weeks	Subgroup A (4 men)	Subgroup B (4 men)	Subgroup A (4 men)	Subgroup B (4 men)		
	Normal diet	Normal diet + extra citrus fruit + 300 mg. ascorbic acid daily	Normal diet	Normal diet + extra citrus fruit + 300 mg. ascorbic acid daily	Normal diet + placeboes	Normal diet + 75 mg. ascorbic acid daily

normal control'' subject received at each meal one capsule containing 25 mg. of ascorbic acid, and each ''normal control'' a placebo identical to the vitamin capsule in size, shape, color, weight and taste. The ''deficient'' subjects lived on a diet devoid of vitamin C, but adequate in other respects, prepared as described below, and received at each meal a placebo. Each ''supplemented deficient'' subject lived on the deficient diet, and received at each meal a capsule containing 25 mg. of ascorbic acid.

It was found possible to destroy all of the ascorbic and dehydroascorbic acids in milk, potatoes, jams, jellies, and yellow vegetables by the addition of 10 ml. of 3% H_2O_2 per 100 gm. of foodstuff followed by 2 hours' steaming in a metal stockpot, with frequent stirring by means of copper rods. The deficient diet provided for breakfast, cereal, vitamin C free milk, coffee, egg dishes or pancakes, toast, oleomargarine and vitamin C free jam; and for lunch and supper, soup bread, oleomargarine, jam, some form of well-cooked meat, mashed potatoes, one other vegetable, a dessert, and a beverage such as coffee, tea or vitamin C free milk. The dining room was arranged so that those on the normal diet ate at one set of mess tables, and those on the deficient diet at another.

In the 2 final weeks of control observations (period 3) both normal groups continued their regime as in period 2. Both deficient groups reverted to the normal camp diet and four from each group (subgroup A) received no other vitamin C. This was to demonstrate the recuperative effects of a normal diet. Four others from each group (subgroup B) received a supplement of extra citrus fruit providing over 200 mg. of ascorbic acid daily and in addition capsules providing 300 mg. daily. This was to demonstrate the recuperative effects of massive doses of vitamin C.

In order to guard against any possibility of vitamin B deficiency, all groups including the normal controls received daily 5 gm. of yeast extract fortified with extra riboflavin.[2]

Dietary observations were made meal by meal. All articles of food were furnished in standard portions of known weight or else the subjects weighed the portion eaten. Each subject kept a record book with a page for each day. On one side of each page was recorded every article of the day's food, and on the other the day's activities hour by hour. The intakes of calories, protein, fat and ascorbic acid were estimated for each day from the tables of Bowes and Church ('42) or from our own analytical data. Any article suspected of containing significant amounts of ascorbic acid was analyzed by the method of Evelyn, Malloy and Rosen ('38) for ascorbic acid and that of Bessey ('38) for dehydroascorbic acid.

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[2] This was Yeast Extract Type 3, kindly provided by Standard Brands, Inc. The daily dose provided approximately 5 mg. of thiamine, 5 mg. of riboflavin and 10 mg. of niacin.

Observations on physical efficiency were made weekly by means of the "pack test" of fitness for hard work, as described by Darling, Johnson, Pitts, Consolazio and Robinson ('44). Scores in this test have in our experience correlated well with stamina as displayed in athletic activities, paralleled improvement in training and revealed deterioration accompanying proved inadequate diets. The Forestry project supervisors provided valuable observations on the subjects' efficiency in their jobs.

The following clinical and laboratory observations were made on each subject: (1) Complete routine physical examination during both the normal periods and at the end of the deficient period. (2) Periodic estimation of blood erythrocytes, leucocytes, hemoglobin and differential count by standard clinical methods. (3) Periodic routine qualitative examination of the urine for formed elements, albumin, sugar and acetone by standard methods. (4) Interview once a week to ascertain whether the subject was suffering from any complaint attributable to the diet. (5) Estimation of the ascorbic acid in the serum and in a 24-hour specimen of urine once a week by a modification of the titration method of Farmer and Abt ('36). (6) At the end of the deficient period, ascorbic acid tolerance tests on all of the "normal controls" and "supplemented normal controls" and on certain of the "deficient" and "supplemented deficient" subjects. After collection of a sample of blood and a timed specimen of urine, 1 gm. of ascorbic acid dissolved in 200 ml. of water was drunk. Specimens of blood and urine were collected at 3 and 6 hours after ingestion.

RESULTS

All groups maintained a reasonably good caloric balance in spite of the experimental restrictions and the somewhat unnatural diet provided for the deficient groups (table 2). Minor changes in weight and small discrepancies between calculated caloric intake and body weight are attributable to the vigorous outdoor schedule which most of the subjects undertook.

The intake of vitamin C was in general kept at the levels desired (table 2). In the final control period, the normal camp diet fell somewhat below the desired 75 mg. a day, owing to a temporary shortage of fruit.

TABLE 2

Body weight, daily caloric intake and daily vitamin C intake.

SUBJECT	JOB [3]	MEASUREMENT									
		BODY WEIGHT END OF PERIOD [1]			CALORIC INTAKE [2] PERIOD [1]			VITAMIN C INTAKE [2] PERIOD [1]			
		1	2	3	1	2	3	1	2	3	
		kg.	*kg.*	*kg.*	*cal.*	*cal.*	*cal.*	*mg.*	*mg.*	*mg.*	
					Deficient group					Subgroup	
										A	B
W.H	Fa	75.1	75.1	...	4590	4330	....	114	0	..	...
W.K.	K	65.7	66.3	66.5	3710	3370	3000	74	0	49	...
R.S.	Fo	77.8	75.0	76.4	3680	3050	3330	62	0	47	...
E.S.	L	62.6	62.0	62.2	2370	1800	2060	100	0	72	...
F.W.	Fo	72.9	69.5	...	3300	3000	....	73	0	..	...
N.H.	K	66.5	65.0	66.2	3490	3170	3060	100	0	..	553
L.M.	O	70.0	69.6	71.0	3140	3300	3120	76	0	..	509
M.R.	R	82.3	79.8	81.6	3620	3490	3230	85	0	..	571
Average		71.6	70.3	70.7	3490	3190	2960	86	0	56	544
					Supplemented deficient group						
W.R.	Fo	70.7	70.3	72.1	3260	3480	3130	79	75	80	...
R.S.	Fo	97.8	97.0	98.2	3570	4180	4290	78	75	82	...
R.W.	Fo	66.4	66.2	66.7	3110	2980	2850	70	75	71	...
B.W.	R	83.4	82.6	82.5	2950	2900	3170	72	75	54	...
J.C.	C	72.6	71.5	71.3	2980	3170	3750	61	75	..	646
J.L.	K	73.5	72.5	74.5	2120	2970	2440	72	75	..	531
M.P.	O	61.5	62.6	63.2	2770	3250	2960	124	75	..	514
F.S.	Fa	70.0	67.4	68.6	4840	4830	4190	98	75	..	631
Average		74.5	73.8	74.6	3200	3470	3350	82	75	72	582
					Normal control group						
W.C.	Fo	71.1	70.5	..	2830	3870	....	106	96	..	
K.K.	M	76.5	73.6	74.3	2990	2880	2860	109	95	59	
W.S.	O	69.5	66.6	68.2	3580	3380	3050	76	83	46	
R.T.	R	73.8	74.6	74.4	3040	3270	3190	52	79	58	
Average		72.7	71.3	72.3	3110	3350	3030	86	88	54	
					Supplemented normal control group						
H.H.	T	71.3	71.8	72.3	3140	3440	3300	100	151	129	
L.L.	K	77.0	75.6	77.0	3080	3100	2780	37	134	125	
T.M.	Fo	69.5	69.5	70.2	3550	3300	2970	83	172	169	
R.N.	K	71.3	69.7	...	2460	2170	....	42	134	...	
Average		72.3	71.7	73.2	3060	3000	3020	66	148	141	

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[1] The experimental periods were: preliminary control, 2 weeks; modified diet, 8 weeks; and final control, 2 weeks.

[2] Calculated from weight of food consumed, analysis of diet and tables. Figures are the averages of all days in a given period.

[3] C, carpentry; Fa, farming; Fo, forestry; K, kitchen; L, laundry; M, camp maintenance; O, office work; R, road construction and repair; T, truck driving.

The physical efficiency of the subjects showed no unusual changes, and none of them complained at any time of symptoms attributable to the diet. They did their daily work efficiently and the Forestry Department supervisors detected no unusual changes in the course of the experiment. Physical fitness as measured by the "pack test" (table 3)

TABLE 3

Physical fitness for hard work. (Scores in "pack test.")

SUBJECT	PERIOD					
	Preliminary control	Experimental				Final control
	Week	Week				Week
	2	2	4	6	8	2
Deficient group						
W.H	85	85	101	..	..	..
W.K.	74	77	83	90	83	83
R.S.	76	76	75	..	..	81
E.S.	92	100	91	91	85	95
F.W.	75	78	81	..	..	..
N.H.	78	80	81	81	88	95
L.M.	73	77	80	91	95	95
M.R.	58	58	69	78	70	66
Average change [1]		0	+ 5	+ 8	+ 3	+ 8
Supplemented deficient group						
W.R.	87	94	106	112	111	108
R.S.	52	50	71	68	70	53
R.W.	102	94	106	94	105	90
B.W.	36	32	31	27	38	46
J.C.	92	93	98	107	104	109
J.L.	84	90	89	96	86	79
M.P.	..	..	82	80	82	79
F.S.	85	83	89	95	104	94
Average change [1]		− 2	+ 6	+ 7	+ 10	+ 6
Control group						
W.C.	71	78	72	..	..	..
K.K.	50	61	58	65	68	52
W.S.	82	81	73	85	73	91
R.T.	..	67	85	86	85	92
H.H.	105	99	110	103	102	106
L.L.	31	40	81	88	68	69
T.M.	78	86	102	87	88	99
R.N.	58	47	31	53	61	..
Average change [1]		− 6	+ 1	+ 6	+ 3	+ 9

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[1] These figures were obtained by setting for each individual a standard score equal to his best in the first three tests he performed, by calculating for each later score the difference from his standard, and then by averaging the individual differences for each test.

showed no significant deterioration in the deficient groups as compared with the controls. In spite of some irregularities in the data, all groups improved somewhat, and a few individuals in each group in striking fashion. These changes are most probably to be attributed to improved physical condition brought about by an active outdoor life. The small drop of the deficient group in the eighth week is paralleled by a similar drop in the control group, and both improved again to the same extent in the final control period. Irregularities in individual scores were in some cases due to changes in motivation from week to week and in others to local disabilities from minor injuries.

TABLE 4

Serum and urinary ascorbic acid. (All figures represent group averages.)

MEASUREMENT	PERIOD						
	Preliminary control	Experimental				Final control	
	Week	Week				Week	
	2	2	4	6	8	2	
		Deficient group				Subgroup	
						A	B
Serum (mg./100 ml.)	0.5	0.6	0.2	0.1	0.0	0.5	1.4
Urine (mg./24 hrs.)	16	20	10	9	9	16	683
		Supplemented deficient group					
Serum (mg./100 ml.)	0.9	1.2	0.8	0.8	0.6	1.0	1.7
Urine (mg./24 hrs.)	22	32	18	18	21	23	850
		Normal control group					
Serum (mg./100 ml.)	0.6	0.9	0.6	0.8	0.8	0.8	
Urine (mg./24 hrs.)	15	23	19	25	19	23	
		Supplemented normal control group					
Serum (mg./100 ml.)	0.7	1.0	1.1	1.1	1.1	1.1	
Urine (mg./24 hrs.)	25	41	50	65	69	93	

In contrast to the lack of change in physical effciency were the marked chemical changes (tables 4 and 5). In the "deficient group" serum ascorbic acid was zero in all but one subject by the end of the fifth week, and in his case it reacted zero by the end of the seventh week. Urinary ascorbic acid dropped to low levels. It is unlikely that the titration method would ever show zero values in urine because of reducing substances other than ascorbic acid, which probably do not disappear during a deficiency of ascorbic acid (Rosenberg, '42). Load tests revealed low body stores at the end of 4 weeks, and complete retention of the test dose by the end of the eighth week. Even after 2 weeks of normal diet, two previously deficient subjects had lower stores than the normal controls. In the "supplemented deficient group" serum and urinary levels and load tests revealed body stores about equal to those of the normal controls. The slight progressive fall in serum ascorbic acid (table 4) was entirely accounted for by one subject's (R.S.) drop to a level of 0.2 mg. per 100 ml. in 8 weeks. However, his urinary excretion and load test were as high as in other members of his group. He was one of the heaviest of all subjects, weighing 97 kg. The "supplemented normal control group" showed the expected high serum levels, urinary levels and load tests. In the present type of oral load test a specimen of blood at 3 hours and collection of urine for the same period would have given essentially the same information as two specimens of blood in 6 hours and collection of urine for the same period.

TABLE 5

Ascorbic acid tolerance tests. (All subjects received 1 gm. of ascorbic acid by mouth.)

NO. OF SUBJ.	DIETARY HISTORY BEFORE TEST	SERUM ASCORBIC ACID Before	SERUM ASCORBIC ACID At 3 hrs.	SERUM ASCORBIC ACID At 6 hrs.	EXCESS ASCORBIC ACID EXCRETED[1] In 3 hrs.	EXCESS ASCORBIC ACID EXCRETED[1] In 6 hrs.
			mg./100 ml.		*mg.*	*mg.*
		Deficient group				
2	Deficient 4 weeks	0.1	0.9	0.7	1	2
2	Deficient 8 weeks	0.0	0.9	0.8	0	0
2	Deficient 8 weeks, then normal diet 2 weeks	0.5	2.7	2.6	16	71
		Supplemented deficient group				
3	Deficient diet 8 weeks, supplemented with 75 mg. of ascorbic acid daily	0.8	2.9	2.3	84	215
		Normal control group				
4	Normal diet 10 weeks	0.8	2.1	1.9	72	262
		Supplemented normal control group				
3	Normal diet 10 weeks, supplemented with 75 mg. of ascorbic acid daily	1.2	2.6	2.2	120	486

[1] This was calculated by estimating the total excretion in the given period and by subtracting from this the excretion for an equivalent time before the test dose of ascorbic acid was ingested.

In no group or subject was there a significant change in erythrocyte count, leukocyte count, hemoglobin or differential count throughout the experiment.

Physical examination revealed changes in only two of the twenty-four subjects in the course of the experiment. One of these (L.M.) was in the "deficient group", the other (M.P.) in the "supplemented deficient group". Subject L.M. had a congenital anomaly of the teeth in the form of three persistent primary incisors. Within 4 weeks of starting the deficient diet his serum level was zero. At the end of 8 weeks his gums, which had been normal to inspection, palpation and pressure in the preliminary control period, showed red margins, bogginess and easy bleeding on pressure. All of these changes were reversed within 10 days when his diet changed to a daily intake of over 500 mg. of ascorbic acid. Subject M.P. had an exacerbation of a previous case of Vincent's angina, which was treated with a course of bismuth. His gingivitis persisted throughout the experimental period, although it did improve slowly.

DISCUSSION

It would appear that in the case of healthy young men on a previously good diet, total deprivation of vitamin C for a period of 8 weeks has no effect on physical efficiency and produces no untoward symptoms. It may in a few individuals lead to minimal changes in the gums and does in all subjects produce marked chemical unsaturation. It would further appear that 75 mg. daily maintains ample body stores of the vitamin for 8 weeks, and that intakes of over 100 mg. daily are of no demonstrable benefit other than to increase the body stores. Our conclusions must not be extended beyond the limits of the experiment, namely 8 weeks, nor be applied to populations different from the one studied. Our data are restricted to normal, healthy young men doing their usual day's work in a temperate environment. Our subjects were deficient in ascorbic acid but they received adequate calories and ample amounts of the other essential nutrients. Their situation was perhaps not exactly comparable to that of populations subject to multiple deficiencies all at the same time.

The subject of Rietschel and Mensching ('39) was deficient for 100 days without a detectable change in efficiency, and the first symptoms of the subject of Crandon, Lund and Dill ('40) occurred not earlier than 60 days after the experimental diet was instituted. The onset of acute deficiency in healthy individuals is therefore apparently followed by clinically detectable scurvy in not less than 8 weeks and detectable inefficiency does not develop in this period. The practical implications for emergency feeding are that vitamin C is somewhat less critical than certain other factors.

SUMMARY

1. If the previous diet has been good, total deprivation of vitamin C for 2 months does not lead in manual workers to detectable deterioration in physical vigor, to inefficiency in the day's work, or to unpleasant symptoms, provided the daily diet is adequate in all nutrients other than vitamin C. Such deprivation may occasionally lead to minimal changes in the gums, and does produce severe desaturation as measured by serum and urinary levels of vitamin C and by tolerance tests.

2. When given in doses of 25 mg. 3 times daily, 75 mg. of ascorbic acid a day appear adequate to maintain or even to increase the body stores of the vitamin in a majority of men held for 2 months on a diet totally deficient in ascorbic acid.

3. Supplements of 75 mg. of ascorbic acid a day when added to a good normal diet are of no detectable benefit to manual workers over a period of 2 months with respect to general well being, physical vigor for hard work, and efficiency in the day's work. Such supplements do lead to increased stores of vitamin C as evidenced by serum and urinary levels and by tolerance tests.

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THE EFFECT OF CHAIN LENGTH OF THE DIETARY FATTY ACID UPON THE FATTY LIVER OF CHOLINE DEFICIENCY[1]

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The observation of Channon and collaborators ('36, '42) that the severity of the fatty liver in choline deficiency was influenced by the nature of the dietary fat has introduced a new factor into the fatty liver problem. The variable that was considered in their studies was the degree of unsaturation of the dietary fatty acids, and the results clearly show that the more saturated the fat of the diet, the more intense was the fatty infiltration of the liver.

In the present study the fatty acids of the diet were varied with respect to their chain length. The effect of each member of the homologous series of even-numbered, saturated fatty acids, from butyric to stearic, has been investigated. These acids have been fed, as ethyl esters, to young rats, at a level of 35% of the diet. The diet contained, in addition, 15% of casein and purified vitamin supplements. Choline was not included, however. After 2 weeks the animals were killed and their livers analyzed for fatty acids.

It will be seen (table 1) that with decreasing chain length of the dietary fatty acids from 18 to 14 carbon atoms, the mean daily weight gain of the rats increased slightly even though less food was consumed. A striking progressive increase in the fatty acid content of the livers is also apparent. When ethyl stearate was fed, normal values for liver fat were obtained. About three times as much fatty acid was recovered from the livers of the animals fed ethyl palmitate and about five times as much from the livers of the rats fed ethyl myristate. Evidence that this increasing fattiness of the livers was due in part to direct deposition of the dietary fatty acid is found in the progressive decrease in the equivalent weight of the liver fatty acids which were isolated.[3]

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[1] This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.

[2] William J. Gies Fellow, 1943–45, University of the Philippines Fellow, 1941–45.

[3] It has been suggested by the editors that this increasing fattiness of the livers may be ascribed to the greater digestibility of the esters of the shorter fatty acids.

In these first groups of animals only one death occurred prior to the fourteenth day. When ethyl laurate was fed, however, an unexpectedly high mortality was encountered. Of the sixteen animals placed on this diet, only one survived the 14-day experimental period. All the others died between the third and the sixth days. The possibility of a toxic contaminant in the lauric acid was excluded by repetition of the experiments with rigorously purified samples. The cause of death was found to be a widespread interstitial myocarditis with necrosis of heart muscle cells. A description of this unusual lesion and studies of its prevention are reported elsewhere (Kesten, Salcedo and Stetten, '45). It is obviously not valid to compare the quantity of liver fatty acid found in rats fed ethyl laurate with the corresponding values obtained after the feeding of other esters not merely because only one rat survived, but, more important, because these rats were suffering from a heart disease peculiar to this group. As there is no satisfactory information relating the state of heart failure to the quantity of liver fat, such a comparison would only serve to confuse the picture.

TABLE 1

The effect of the chain length of the dietary fatty acid upon the quantity of liver fat.

Young male rats were placed on choline-free diets containing 35% of the ethyl esters of various fatty acids. The livers of the rats surviving 14 days were analyzed for fatty acid content.

FATTY ACID OF DIET	NO. OF RATS	MEAN DAILY FOOD CONSUMPTION	MEAN DAILY WEIGHT GAIN	DEATHS	LIVER FATTY ACIDS		MEAN EQUIVALENT WEIGHT OF LIVER FATTY ACIDS
					Mean	Spread	
		gm.	*gm.*		*% of liver*		
Stearic	4	7.5	1.2	1(14) [1]	3.2	2.3– 4.0	289
Palmitic	4	7.3	1.3	0	9.4	6.0–14.4	275
Myristic	4	5.3	1.6	0	17.2	15.5–22.4	266
Lauric	16	3.9	0.5	15(4.4)	8.0	..	261
Capric	2	6.0	1.6	0	7.2	7.1– 7.2	289
Caprylic	9	5.2	1.9	4(9.8)	4.4	2.8– 7.4	260
Caproic	2	7.0	1.2	0	7.0	6.5– 7.4	267
Butyric	2	4.0	..	2(2)	..	..	..
None	4	11.2	3.3	0	7.1	3.6–10.8	276

[1] The figures in parentheses are the average survival times, in days, of the animals which died.

With fatty acids shorter than lauric acid no severe fatty livers were obtained. Several deaths occurred in the group of rats receiving ethyl caprylate. The chief pathological finding in this group was renal hemorrhage and necrosis, similar in distribution to that described previously as occurring in choline deficiency (Engel and Salmon, '41). Curiously, these kidney lesions were observed in none of the animals receiving other fatty supplements except in one that had been fed ethyl laurate.

The feeding of ethyl butyrate resulted in death within 2 days. At autopsy, the intestinal tracts of these animals were found to be full of blood, and microscopic examination revealed acute gastric ulceration as the site of hemorrhage.

To a control group of rats a diet devoid of fatty supplement was fed, the carbohydrate of the diet being correspondingly increased. These animals consumed more food and gained more in weight than did any of the other groups. They developed mild fatty livers and the equivalent weight of the liver fatty acids was almost the same as when palmitic ester was fed. This is in accord with the view that it is predominantly the 16-carbon fatty acid that is synthesized by rats on a high carbohydrate diet (Longenecker, '39).

Our results, considered together with those of Channon et al., ('36, '42), show that the response that may be expected to a diet devoid of lipotropic methyl will be critically dependent upon the nature of the fat in the diet. The resulting fattiness of the liver will be influenced not only by the degree of unsaturation of the dietary fat but also by the molecular weights of the constituent fatty acids. It is possible that some of the discrepancies observed by several workers in the fatty liver problem are due to unrecognized variations in the composition of the dietary fat.

EXPERIMENTAL

Samples of stearic, palmitic, myristic and lauric acids[4] were esterified with ethyl alcohol in the usual fashion and the products distilled in vacuo through a Vigreux column. Commercial samples of lauric acid and ethyl laurate,[5] were also used. One portion of lauric acid was subjected to careful preliminary purification by solution in warm conc. H_2SO_4, precipitation by dilution with water, followed by repeated recrystallization from aqueous acetone. The lower fatty acids were all commercial products.[6] The purity of each fatty acid was controlled by determination of its titration equivalent weight and iodine number.

The diet used contained 35% of fatty acid ethyl ester, 15% of casein,[7] 44% of glucose monohydrate, 4% of salt mixture no. 2, U.S.P. XII and

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[4] The authors wish to express their gratitude to Mr. H. Sherman of the Massachusetts Institute of Technology for his gift of these samples.

[5] Samples of lauric acid and ethyl laurate were purchased from the Eastman Kodak Company.

[6] Lower fatty acids were purchased from the Eastman Kodak Company.

[7] Labco, vitamin-free.

2% of roughage.[8] To each milligram of this diet were added 6.25 mg. of thiamine hydrochloride, 5 mg. each of riboflavin and pyridoxine, and 25 mg. each of niacin and calcium pantothenate. The fat-soluble vitamins were given by mouth to each animal in the following doses: 1,700 units of vitamin A and 200 units of vitamin D each week, and 0.2 ml. of linseed oil every 10 days. In the diet of that group of rats that received no fatty acid ethyl ester, the carbohydrate fraction was increased from 44 to 79%.

Male rats of the Sherman strain, about 5 weeks old and weighing 55–60 gm. were used throughout. The animals which survived the 14-day period were killed by asphyxiation with N_2. Their livers were individually digested with alkali and fatty acids isolated in the usual fashion (Stetten and Schoenheimer, '40). The quantities of fatty acid tabulated (table 1) are averages of individual determinations. The liver fatty acids of each group were pooled and their mean equivalent weights determined by titration with 0.1 N alkali in 50% ethanol.

SUMMARY

Each member of the homologous series of even-numbered fatty acids, from butyric to stearic, has been fed as ethyl ester, at a 35% level in the diet, to young male rats receiving no choline.

The degree of fatty liver was found to increase markedly as the chain length of the dietary fatty acid decreased successively from 18- to 16- to 14-carbon atoms. No severe fatty livers were encountered when the ethyl esters of fatty acids of less than 12-carbon atoms were fed.

Renal hemorrhage and necrosis occurred in several of the rats fed ethyl caprylate and a fatal myocarditis was encountered when ethyl laurate was fed.

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[8] Celluration.

FATAL MYOCARDITIS IN CHOLINE DEFICIENT RATS FED ETHYL LAURATE [1]

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FOUR FIGURES

(Received for publication September 29, 1944)

In the course of a study of the relationship of chain length of dietary fatty acids to fatty liver (Stetten and Salcedo, '45), an unexpected mortality was encountered when rats on a choline-free diet were fed the ethyl ester of lauric acid. It was observed that of a total of sixteen rats on a synthetic diet containing 35% of ethyl laurate, all but one died between the third and sixth days. Prior to death these animals exhibited no untoward symptoms although they consumed less food than did rats receiving the ethyl esters of homologous fatty acids. Animals were observed to be normally active within 2 hours prior to death.

That congestive heart failure was the cause of death was clear from the pathological findings. The hearts were small, and the ventricles pale and in systole. Effusions in the body cavities were occasionally noted.

Histological examination revealed the cause of the heart failure to be a widespread interstitial myocarditis associated usually with necrosis of isolated muscle cells. In some cases the myocardium of all the chambers was involved in a patchy fashion; in others, only that of the ventricles. The subendocardial regions seemed especially susceptible (figs. 1 and 2) but the conduction system was generally not strikingly involved nor were thrombi formed on the overlying endocardium.

The interstitial tissue was spread apart and infiltrated by wandering cells that included large mononuclears, lymphocytes and neutrophilic polymorphonuclears, but macrophages and lymphocytes predominated (fig. 3). A few cells resembling plasma cells were also present. The cytoplasm of the large mononuclear wandering cells was devoid of phagocytized material. Fibrin was not present, but a mild degree of

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[1] This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.

[2] William J. Gies Fellow, 1943–45; University of the Philippines Fellow, 1941–45.

edema was suggested by the presence of a little fine eosinophilic granular material about the wandering cells. Giant cells were not found.

The extent of visible damage to the heart muscle cells was variable. In the more severe cases a considerable number of swollen necrotic cells were found, singly, or in small groups of a few cells (fig. 4). The cytoplasm was altered in a segmental manner, with loss of cross-striations, and pallor of the sarcoplasm. In the less severely affected hearts, histologically demonstrable damage to the muscle cells was not always seen, although the interstitial inflammatory reaction was present, as indicated by small groups of wandering cells at many points. No evidence of regeneration of muscle or of proliferation of connective tissue was observed in any of the animals, nor could any undue accumulation of cardiac fat be demonstrated histologically.

Except for the fatty livers, the other organs of the rats exhibited no microscopic alterations beyond congestion, this being sometimes quite marked in the liver.

It should be pointed out that these cardiac changes, in our series, appear to be specificially related to the ingestion of lauric acid. We have fed, as ethyl ester, each member of the homologous series of even-numbered saturated fatty acids to rats and in only one animal receiving a fatty acid other than lauric were myocardial inflammatory changes observed. This rat, fed ethyl stearate, died on the fourteenth day of feeding, and this isolated case may well be unrelated etiologically to the disease which results from lauric acid feeding.

In view of the inflammatory nature of the cardiac lesion, bacterological studies have been carried out. Heart tissue was taken for culture from three rats by Dr. H. Rose of the Department of Medicine. These rats had been sacrificed 4, 5 and 6 days, respectively, after being placed on the ethyl laurate diet. The cultures showed no growth on aerobic or anaerobic media. Blood smears have also been examined for parasites but none was found.

In a further study of the myocardial lesion, the concentration of ethyl laurate in the diet has been varied (table 1). Whereas a 40% level proved uniformly fatal, a 25% level was tolerated. Even in these

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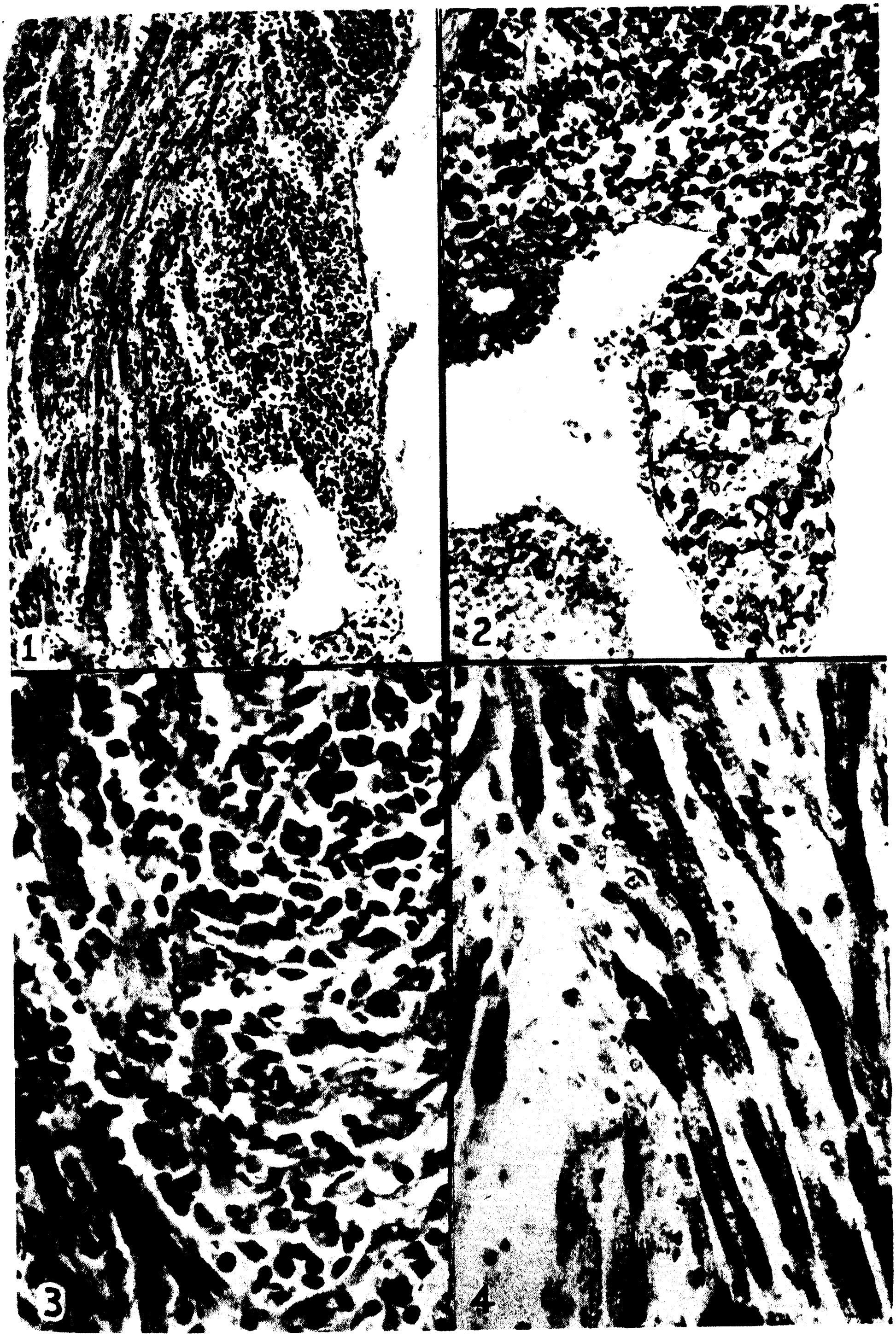
Fig. 1 Rat 44–44. Interstitial myocarditis in wall of right ventricle, especially near endocardial surface (× 120). Ethyl laurate 35%. Death on fourth day.

Fig. 2 Rat 44–19. Subendocardial and interstitial round cell infiltration. (× 300). Purified ethyl laurate 35%. Death on fourth day.

Fig. 3 Rat 44–44. Acute interstitial myocarditis with fragmentation of muscle cellls in wall of right ventricle (× 460).

Fig. 4 Rat 44–44. Degeneration and necrosis of myocardial cells. (Mallory's phosphotungstic acid hematoxylin stain. × 460).



Figures 1 to 4

animals, however, histological evidence of diffuse interstitial myocarditis was found, though to a much milder degree than in the rats fed 35% of ethyl laurate. The addition of 0.5% of *l*-cystine to a diet containing 25% of ethyl laurate appeared to aggravate the pathological process in the heart slightly. The degree of fatty liver in these rats was more marked.

TABLE 1

Effect of various supplements upon rats receiving ethyl laurate.

Ethyl laurate and various supplements were incorporated into the diets of young male rats in the concentrations indicated. The livers of the rats surviving 14 days were analyzed for fatty acids.

ETHYL LAURATE	SUPPLEMENT	NO. OF RATS	MEAN DAILY FOOD CONSUMPTION	MEAN DAILY WEIGHT GAIN	DEATHS	LIVER FATTY ACIDS	MEAN EQUIVALENT WEIGHT OF LIVER FATTY ACIDS
% of diet	*% of diet*		*gm.*	*gm.*		*% of liver*	
35	..	16	3.9	0.5	15(4.4) [1]	8.0	261
40	..	4	..	..	4(5.5) [1]	..	..
25	..	2	5.5	0.9	0	15.0	254
25	*l*-Cystine, 0.5	2	4.6	1.3	0	20.6	257
35	Choline chloride, 0.5	4	6.0	1.7	0	4.0	256
35	Inositol, 0.5	2	2.6	−1.2	2(5.5) [1]	..	..
35	Betaine hydrochloride, 1.25	2	3.5	+0.6	2(6.5) [1]	..	..
35	Betaine hydrochloride, 2.5	2	5.3	2.0	0	3.8	256
35	*dl*-Methionine, 1.25	2	3.6	−0.2	1(4) [1]	7.8	259
35	*dl*-Methionine, 2.5	2	4.5	+1.2	0	2.8	260
35	K_2HPO_4, 3.5 [2]	6	2.9	−0.96	6(4.3) [1]	..	..

[1] The figures in parentheses are the average survival times, in days, of the animals which died.

[2] The stock diet contains 0.96% K_2HPO_4.

A few studies of the prevention of the cardiac lesion have been completed. The addition of 0.5% of choline chloride to a diet containing 35% of ethyl laurate completely prevented the appearance of myocardial changes. The same concentration of inositol was devoid of any prophylactic action. Betaine hydrochloride, incorporated in the diet at a level of 1.25% did not prevent the disease, but at twice this concention, it was completely effective. Likewise, *dl*-methionine was only partially prophylactic at a level of 1.25%, but at 2.5% prevented the appearance of any cardiac changes. The expense of the purified fatty acids precluded the use of large series of animals. The prevention of the cardiac lesion by these "lipotropic agents" was sufficiently dramatic in the small groups of animals used, however, to warrant its inclusion in the present paper.

In their description of the pathological findings in rats on choline deficient diets. Engel and Salmon ('41) incidentally mention myocardial changes. As these authors were not primarily concerned with variations in dietary fat, it is difficult to say whether they were observing the same disease as we have described. In the lesions that they observed, myocardial hemorrhage is prominent, a phenomenon not encountered in our series.

Follis, Orent-Keiles and McCollum ('42) have described cardiac changes, very simliar histologically to those encountered in the present experiments, which occurred when diets extremely poor in potassium were fed. Their disease was characterized by survival for 6–47 weeks, by complete prevention when the level of K in the diet was raised to 0.44%, and by a decrease of some 30% in the K content of the heart. Our rats normally received 0.43% of K in their diets. Further increase of the dietary K to a value of 2.0% did not interfere with the development of myocarditis or the fatal income. The possibility that our ethyl laurate diets caused a depletion of K in the heart muscle cells has been ruled out by potassium analyses on the hearts of rats which had been fed the disease-producing diet for 4 days. The addition to the diet of choline, which has been shown to prevent the disease, did not significantly alter the myocardial K content (table 2).

A cardiac failure incident to an acute interstitial myocarditis, fatal in about 4–5 days, may thus be provoked in young rats on a diet poor in lipotropic agents, if 35% or more of the diet is supplied as ethyl laurate. The disease, quite specifically related to the 12–carbon fatty acid, appears to be due neither to a toxic contaminant nor to a bacterial or parasitic agent. It must therefore be supposed to be related to some property of lauric acid itself. It is perhaps worth noting that certain other biological peculiarities of lauric acid have been reported in the literature (Siegler and Popenoe, '24; Ozaki, '27; Powell, '30).

EXPERIMENTAL

The ethyl laurate used in these experiments was prepared and purified as described in the preceding paper. When it was fed at a 35% level, the remainder of the basal diet was the same as that previously described. In the experiments in which ethyl laurate was fed at a 25% level, the carbohydrate of the diet was increased to 54%. To one group of animals ethyl laurate was fed at a 40% level. In this case, the casein was cut to 10% of the diet. The vitamin supplements were given in all cases as mentioned in the previous report.

Additional supplements of choline chloride, meso-inositol, l-cystine, dl-methionine, betaine hydrochloride and K_2HPO_4 were added to diets in concentrations indicated in table 1.

TABLE 2

Potassium content of hearts and kidneys of rats fed ethyl laurate.

Two groups of 3 rats each were kept for 4 days on diets containing 35% of ethyl laurate. Choline chloride, 0.5%, was incorporated in the diet of the control group. Pooled hearts and kidneys from each group were analyzed for K.

		FRESH WEIGHT	DRY WEIGHT	K	K	K
		mg.	*mg.*	*mg.*	*% of dry weight*	*% of ash*
No choline added	Hearts	649	133	2.43	1.82	41
	Kidneys	1852	416	4.87	1.17	21
Choline added	Hearts	631	131	2.56	1.95	43
	Kidneys	1667	376	4.31	1.15	21

Two groups of three rats each were placed on the 35% ethyl laurate diet. To the diet of one group, 0.5% of choline chloride was added. After 4 days, the rats were killed, and the hearts and kidneys then removed. The pooled hearts and the pooled kidneys from the rats of each group were dried to constant weight of 100°C., ashed (Stolte, '11), and the ash in each case analyzed for K (Shohl and Bennett, '28) (table 2).

All the remaining operations were carried out as previously described (Stetten and Salcedo, '45).

SUMMARY

The feeding of ethyl laurate, at a 35–40% level in the diet, to young rats receiving no choline resulted in death from heart failure in from 3 to 6 days. The pathological process was an acute diffuse interstitial myocarditis. It was preventable by the adequate administration of choline, betaine or methionine. The disease did not occur in rats fed the ethyl esters of other fatty acids and was not fatal when the quantity of ethyl laurate was reduced to 25% of the diet. Though histologically similar, the disease is apparently not identical with the myocarditis of potassium deficiency.

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OBSERVATIONS ON RIBOFLAVIN EXCRETION BY THE ADULT MALE[1]

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ONE FIGURE

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If the requirement of the human adult for riboflavin is as high as suggested by the Food and Nutrition Board of the National Research Council ('43), recent surveys (Bing, '43) indicate that riboflavin deficiency is one of the commonest inadequacies in the diet of American laborers. Investigation of the quantitative aspects of this riboflavin requirement was undertaken as a part of the study begun in March, 1942, of the nutritional requirements of adult males (Kyhos et al., '44). Subjects were chosen from male inmates of the Wisconsin State Prison at Waupun; all were in good health, doing moderate to heavy work in prison industries, and had been inmates at least a year. Their co-operation was voluntary. To get data on the riboflavin requirement, it was our intention to determine the basic level of riboflavin excretion and the effect of supplements on riboflavin excretion; to note sources of riboflavin in the diet; and to conduct periodic physical examinations to observe any clinical signs that could be correlated with variations in intake and excretion. These observations extended over a period of 2 years.

Physical examinations were limited to inspection of the sclerae, skin about the eyes, nose and mouth, the lips, tongue and gums. A portable slit lamp providing about 4 diopters magnification was used.

DIET

Details of the prison diet have been given in a previous report (Kyhos et al., '44). It is high in carbohydrates and muscle meats, low in fresh fruits and fresh vegetables. Butter is served once a day, eggs and cheese each twice a week. Milk is used in cooking various foods, and is mixed with the coffee before serving. The recipe calls for 10 pounds cream and 40 pounds whole milk in 100 gallons of coffee, i.e., 1 part in 16. Three times a week cereal and milk are given at breakfast, and the men may drink as much milk as they want at this time. Liver is served at irregular intervals, never oftener than once a month.

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[1] This project has been made possible by special grants from the Wisconsin Alumni Research Foundation at its inception, and has been supported chiefly by the assistance of the Nutrition Foundation, in a grant to one of us (E.L.S.).

It was not practicable to keep the subjects on a measured diet, or to determine the amount of riboflavin in the food as actually served and consumed. From inspection of the menus it appeared that milk would be the chief source of riboflavin, and that all the men had the opportunity to drink 2 or 3 cups of milk three times a week. Some were given extra milk for various reasons, and some used their earnings to buy extra fruit, candy, peanuts and vitamin preparations.

METHODS

The microbiological method of Snell and Strong ('39) was used. All analyses were done by the same operator. Excretion was determined by assay of 24-hour urine samples, collection beginning at 6 A. M. For protection against light, alkaline urine, and bacterial decomposition, dark brown glass bottles were used, containing as preservative 10 ml. glacial acetic acid, 1 ml. toluene, and 1 ml. chloroform. Bottles were kept cool in a dark container, and taken by truck the next morning to the laboratory at Madison, 55 miles away. On arrival, samples were checked to insure acidity, and refrigerated under toluene. A total of 360 24-hour samples was examined. Samples were assayed within 1–3 days after collection. On 180 of the samples, analysis was repeated within 3 weeks, and no significant variation was noted in riboflavin content: 120 showed less than 10% variation; of the remaining 60 none varied more than the limit of 20% ascribed to the method by the authors.

RESULTS

Preliminary survey

Single samples from 68 men ranged in riboflavin content from 0.05 to 2.4 mg. No correlation appeared between this variation and the ages and weights of the men. Ages ranged from 19 to 59, weights from 130 to 218 lbs. It will be readily understood that in such an institution detailed investigation cannot be undertaken with all types of inmates. On the other hand, institutional regulations prevent indiscriminate access to food. Purchases and gifts are each limited to once a month and are a matter of record. Extra or special foods from the prison are obtained on medical recommendation. Intelligence and cooperation on the part of the men also assisted in obtaining definite and reliable information about the food habits of 43 individuals. In table 1 a summary of these observations is presented which emphasizes the correlation between riboflavin excretion and the intake of materials rich in riboflavin: milk, liver, peanuts and vitamin preparations.

TABLE 1

Ranges in riboflavin excretion shown in single 24-hour specimens from 68 men.

TOTAL NO. OF MEN	24-HOUR EXCRETION	FOOD HABITS OF 43 MEN
	mg.	
25	0.05–0.2	7 drank no milk; 7 drank less than 3 cups per week.
27	0.2 –0.5	16 drank 9–10 cups of milk per week (regular diet).
16	0.5 –2.4	13 drank extra milk, ate peanuts, or took vitamin tablets.

Intensive study preliminary to supplementation

Of the 43 men with known food habits, we were able to continue the study of 29, who in different groups furnished 24-hour urine samples once weekly over a period of 15 months. Sunday was selected as the day of collection because of convenience for the prison staff and inmates. The menus for Saturday and Sunday were standard, including cheese once, meat twice daily, and eggs and milk for Sunday breakfast. Urine collection on Sunday and on the following Monday by 16 men showed substantial agreement in each case. One man on the regular diet showed an average excretion in 15 samples of 0.28 mg. He collected 6 consecutive daily samples, with riboflavin content as follows: Sunday, 0.26 mg.; Monday, 0.27 mg.; Tuesday, 0.18 mg.; Wednesday, 0.22 mg.; Thursday, 0.38 mg.; Friday, 0.46 mg.; and on these last 2 days holiday meals including chicken with liver dressing were served. We felt therefore that samples collected on Sunday were fairly representative of the usual excretion.

The 29 men were classified in three groups. Group A consisted of 16 men eating the regular diet which included 9–10 cups of milk per week. In group B there were 5 men who drank no milk and used none on cereal. Group C was made up of 9 men who ate the regular diet plus extra milk, peanuts or vitamin tablets. A total of 108 weekly urine samples were collected by the group of 29. While occasional individual variations occurred from 1 week to another, the average excretion for each man, and for each diet group, showed a correlation with the intake of riboflavin (table 2).

In group A, the individual average excretion (table 2) ranged from 0.2 mg. to 0.47 mg. with a group average of 0.3 mg. Average excretion per month ranged from 0.21 to 0.35, with no seasonal variation. General daily average was 0.3 mg. Again, no correlation with weight or age could be found. In group B, men who drank no milk, the average excretion was 0.1 mg., with a range of 0.05 to 0.12. In group C, excretion varied with the type of extra nourishment taken. That of men taking vitamin tablets was always above 1.0 mg. Men taking an extra pint of milk daily excreted slightly less than 1.0 mg. Those eating peanuts excreted about 0.6 mg. The riboflavin content of these materials, taken from food charts published by the American Medical Association ('42) and from the manufacturer's claims of potency for vitamin preparations are as follows: peanuts 1.76 mg./lb., milk 0.96 mg./pint, liver 3.40 mg./4 oz., vitamin preparation A 2.00 mg./1 tablet, and vitamin preparation B 2.00 mg./3 tablets.

TABLE 2

24-hour excretion of riboflavin in special diet groups.

GROUP	A		B			C			EXTRA SOURCE OF RIBOFLAVIN
No. of men	Av. excr.	Total N	No. of men	Av. excr.	Total N	No. of men	Av. excr.	Total N	
	mg.	*gm.*		*mg.*	*gm.*		*mg.*	*gm.*	
8	0.2–0.3	10.3–14.5	5	0.05–0.12	7.4–12.4	2	1.3, 1.5		Vitamin preparation A
6	0.3–0.4	8.5–15.5				1	1.4		Vitamin preparation B
2	0.4–0.47	13.8				3	0.9, 0.7, 0.5	7.2–14.2	½–1 pt. milk
						2	0.6, 0.56	9.5–11.5	Peanuts

The total nitrogen excretion data in table 2 give an idea of the level of nutrition and especially of the meat intake in these subjects. The range of 7.2 to 15.5 gm. nitrogen excretion indicates that nitrogen equilibrium might well be expected, since these men have access to as much food as they desire at meal time, and were in approximate weight equilibrium. No correlation could be established between the nitrogen excretion and riboflavin in the 24-hour urine samples, probably because muscle meats and cereal products were the chief sources of nitrogen in the diet. There was no tendency to increased riboflavin excretion with low nitrogen levels, as might be expected from the observations of Sarett and Perlzweig ('43) on rats. However their data indicated that in order to obtain riboflavin deposit in the liver the rats must be depositing protein. Thus there is no reason to expect that a moderately low nitrogen level, especially in equilibrium conditions, would tend to increase riboflavin loss.

Results of physical examinations

In group A, using the ordinary prison diet, 14 of the 16 men showed grossly visible corneal injection, usually accompanied by various sizes of yellowish plaques of tissue on one or both sides of the pupil. In this group 5 had tongues with distinct furrows, of varying pattern. But only one man had scars at the canthi of the lips, suggesting former active lesions, and there were none with lesions which could be described as cheilosis.

In group C, of the 8 men who used supplements of various sorts, 4 had similar corneal lesions, 2 had furrowed tongues and a third had an atrophic tongue. It was evident that 3 had scars from old cheilotic lesions, and that a fourth member of the group had suggestive lesions of current disturbance possibly to be called cheilitis.

By contrast the observations of the 5 men in group B, who by taste had been avoiding milk for 2 or more years, showed that only one had the corneal lesions described above. None of these men had a furrowed tongue. Search for the lesions at the canthi of the lips, about the nasolabial folds, or about the eyes led to discovery of only one man with questionable cheilitis at the lips. This was in the individual with the lowest excretion of riboflavin, averaging 50 µg. daily.

Effect of supplements

Although no clinical signs attributable with certainty to ariboflavinosis had been observed, the plan of giving riboflavin supplementation was carried out. Excretion due to supplements of natural sources of riboflavin was compared with that following administration of synthetic riboflavin. Supplements were given for 3 to 6 weeks to groups of 5–6 men each, with collection of 180 weekly urine samples as before. Due to difficulties of various sorts the final groups who completed the studies were composed of 2 to 5 men each. The milk supplement was given between meals. Yeast tablets and tablets of synthetic riboflavin were given after meals, to avoid too rapid absorption and excretion. Each yeast tablet contained 0.4 gm. of dried unfermentable brewer's yeast fortified with a natural riboflavin concentrate from processed corn, furnishing about 0.085 mg. of riboflavin per tablet.[2] Synthetic riboflavin [3] was used in tablets of 0.5, 1.0 and 2.0 mg. content.

Table 3 summarizes the effects of supplementation on the excretion of men in group A (9–10 cups of milk per week). Average excretion was increased 0.2 to 0.28 mg. by supplements of 0.5 mg.; it was increased 0.7 to 0.8 mg. by supplements of 1.0 mg. in the form of milk and yeast, and increased 0.2 mg. by 1.0 mg. of synthetic riboflavin. To illustrate the consistency of the increase: one subject with an average basic excretion of 0.28 mg. excreted 0.50, 0.55, 0.47 and 0.50 mg. in 4 consecutive daily 24-hour samples while taking 1.0 mg. of synthetic riboflavin daily. During the administration of 6 yeast tablets daily, a meal of liver resulted in excretions ranging from 0.87 to 1.34 mg.; these readings were excluded from the averages.

TABLE 3

Retention of riboflavin supplements.

NO. OF SUBJECTS	DAILY SUPPLEMENTS	AVERAGE EXCRETION		SUPPLEMENT RETAINED
		Before	After	
		mg.	*mg.*	*mg.*
4	Milk — 1 cup (0.48 mg.)	0.35	0.63	0.20
4	Milk — 2 cups (0.96 mg.)	0.35	1.15	0.16
3	6 yeast tablets (0.5 mg. riboflavin)	0.30	0.50	0.30
2	12 yeast tablets (1.0 mg. riboflavin)	0.30	1.00	0.30
3	1.0 mg. synthetic riboflavin	0.30	0.50	0.80
5	2.0 mg. synthetic riboflavin	0.30	1.58	0.72

Table 4 gives data on supplementation of group B, men on the regular diet who drank no milk. Their basic excretion (average 0.1 mg.) was raised 0.15 to 0.2 mg. by addition of 0.5 mg. of synthetic riboflavin; it was raised 0.35 to 0.6 mg. by 1.0 mg. daily.

Men in group C, with a high basic excretion due to extra intake of milk responded to supplementation by a further rise in excretion (not shown in tables). For example, a group with an average excretion of 0.7 mg. excreted over 1.0 mg. daily while taking 6 yeast tablets (0.5 mg. riboflavin) daily; a meal of liver during this period produced excretions ranging from 1.36 to 2.84 mg. the following day.

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[2] Fleischmann

[3] Merck.

Probably the significance of these data lies in the amount of supplement retained rather than in the amount excreted. From the data in table 3 it is seen that when milk supplements were used, and assuming the milk content of riboflavin to be 0.96 mg. per pint (Food Charts, '42), the retention was about 41% with one-half pint, but only 17% of the supplement when a pint was taken. This retention is significantly less than that reported by Gardner et al. ('43) on a milk diet yielding 7.0 mg. riboflavin. Using yeast supplements of comparable riboflavin content, the retention was 60% on 0.5 mg. intake, but only 30% on 1.0 mg. When pure riboflavin was given in tablet form, retention of 80% was observed on 1.0 mg., dropping to 36% when the dose was doubled. Data taken from table 4, where the men were on a lower riboflavin intake, indicate that after supplementation with pure riboflavin in 0.5 mg. tablet doses retention averaged 66%, and that when the dose was increased to 1.0 mg. the retention was still at 55%. By the criteria of basic urinary excretion as well as the type of diet used these men were more apt to be unsaturated than any other men we have studied. Yet their retention of riboflavin supplements was not strikingly greater than that of the others on a higher intake.

TABLE 4

Group B — Riboflavin excretion. Before and after supplements of synthetic riboflavin.

SUBJECT NO.	BEFORE SUPPLEMENT		AFTER SUPPLEMENT			
			0.5 mg. supplement		1.0 mg. supplement	
	"Fasting" hour excretion	24-hour excretion	24-hour excretion	Retained	24-hour excretion	Retained
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
22	0.	0.05	0.20	0.35	0.40	0.65
20	0.003	0.10	0.25	0.35	0.50	0.60
23	0.005	0.10	0.25	0.35	0.60	0.50
21	0.007	0.10	0.30	0.30	0.70	0.40
Average		0.10	0.25	0.33	0.55	0.55

These same relations are shown graphically in three curves (fig. 1). The total excretion is plotted as ordinates, the supplemental intake as abscissae. It will be noted that the excretion rises faster than the rate of increase in amount of supplement. This relation was true in every individual case where the subject received two different doses of the same supplement. Such a relationship between intake and excretion makes it probable that riboflavin is a non-threshold substance, excreted by the kidneys even when the intake is very low. The curves connecting the points on these three typical subjects have been extrapolated to the intersection with the base line. This procedure should serve to indicate an approximate intake on the unsupplemented diet, by the distance to the left of the ordinate axis. The two men on the ordinary prison diet appear to be securing about 1.5 mg. riboflavin.

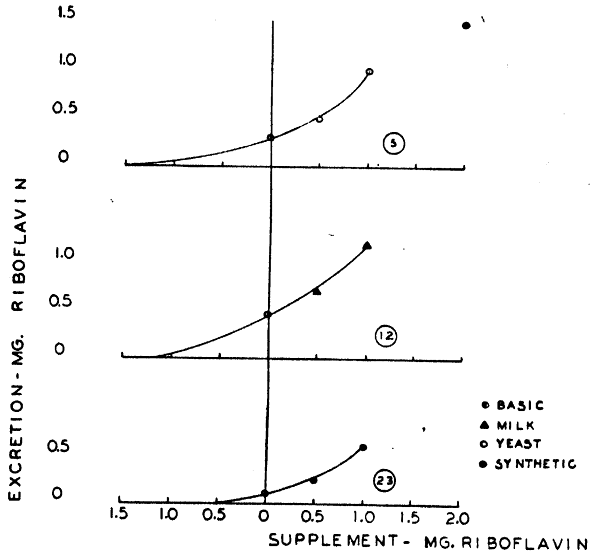

Fig. 1 Rate of increase in riboflavin excretion with diet supplementation. Subject 5, on ordinary prison diet, supplemented with yeast and with synthetic riboflavin. Subject 12, on ordinary prison diet, supplemented with milk. Subject 23, usually avoiding milk, supplemented with synthetic riboflavin.

The one who avoided milk was getting about 0.5 mg. The data from the other members of this group suggest intakes of the same order of magnitude. We are therefore inclined to believe that these men have been subsisting for a period of 2 or more years, as they asserted, on a diet which provides this very small amount of riboflavin. Yet we were unable to detect in them any of the physical findings supposedly characteristic of riboflavin deficiency. Furthermore, after they had been receiving the supplements for periods of several weeks, they gave no consistent reports of any subjective improvement.

In table 4 the riboflavin excretions in 1-hour urine samples, taken just before breakfast, are compared with the excretions in the succeeding 24 hours when no riboflavin supplement was administered. Considering the great errors introduced when the concentrations are so low, the agreement is fair. We are not impressed with the usefulness of such single hour specimens (Holt, '43) for this type of study.

On 180 of the 24-hour urine samples, determination of pantothenic acid was done by the microbiological method of Strong, Feeney and Earle ('41). Individual variations were marked at all seasons. Most of the values were in or near the accepted normal range of 2–5 mg. (Gordon, '42). No correlation was seen with food intake, with riboflavin excretion, or with changes in riboflavin intake. The extreme range of values was 1.1 to 6.6 mg. Pantothenic acid determinations were not done during periods when yeast was administered.

DISCUSSION

The physical findings in our subjects fail to convince us of any correlation between riboflavin intake and such lesions as cheilosis, changes in naso-labial skin, or chronic lesions of the sclerae. In fact such lesions were far more common among men who were on fair or supplemented diets than among those whose long-standing habitual avoidance of milk kept them on the lowest riboflavin intakes we have observed. We consequently must question cheilosis, chronic lesions at the naso-labial folds, or thickened and vascularized patches in the sclerae as being evidences of long continued riboflavin deficiency.

We have no accurate measure of the total riboflavin intake of our subjects. The excretion of riboflavin was demonstrably increased by milk, by other foods such as liver or peanuts, by the use of pure riboflavin in tablet form, or by yeast tablets. The data in tables 3 and 4 indicate that of the various supplements the retention was from 17 to 80%. A variable state of saturation does not appear to be a sufficient explanation for this wide fluctuation. The data are not isolated observations, but are based on averages of excretion at weekly intervals. There was no distinct increase or decrease of excretion in such a series of weekly averages to suggest a marked change in the state of saturation during the period of weeks while a given supplement was being used.

The excretion of 50 to 100 µg. riboflavin by the men in our group B is of the same order of magnitude as that reported by Keys et al. ('44) for their subjects who received 0.82 to 0.90 mg. daily. This circumstance adds to our conviction that our subjects were receiving not much more than 0.5 mg. daily in their conventional intake. Keys et al. show retentions of supplemental administered riboflavin varying from 38 to 90%.

If the intake of much less than 1.0 mg. daily of riboflavin for such long periods as 2 years fails to induce cheilosis or the other lesions formerly attributed to deficiency of this factor, we are led to question the nature of objective data for the need of riboflavin by the adult human. The report of Williams et al. ('43) deals with subjects whose dietary intake was similar to ours except for better supplementation with other vitamins. Their only evidence of riboflavin deficiency was decreased excretion of test doses, i.e., of tissue desaturation. In our opinion this does not demonstrate any need for the vitamin. Doubt about the validity of saturation tests has been expressed by Axelrod et al. ('41). It would be surprising if there were no requirement for a vitamin which is a constituent of an important enzyme system. We believe riboflavin is a vitamin for the human. The urgent need is for evidences from physical examination or from biochemical studies which will demonstrate this need and allow detection of subjects with deficiency. When this end has been achieved the magnitude of the need can be studied.

SUMMARY

Studies made on a group of adult male prisoners showed that after ingestion of riboflavin supplements in the form of tablets or foods rich in vitamins there was an increase of riboflavin excreted in the urine. The retention of riboflavin from such supplements varied from 17 to 80% with no apparent reason for the wide fluctuations. No physical findings were seen to indicate riboflavin deficiency in those men who for 2 years or more must have been eating not much over 0.5 mg. daily, and who were excreting 0.05 to 0.12 mg. riboflavin in the 24-hour urine. It is felt that satisfactory criteria of need for riboflavin by the human adult are still lacking.

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NIACIN (NICOTINIC ACID), AN ESSENTIAL GROWTH FACTOR FOR RABBITS FED A PURIFIED DIET

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FIVE FIGURES

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Hogan and Ritchie in 1934 reported successfully raising rabbits on simplified diets. Their most simplified successful diet consisted of commercial casein 20%; dextrinized cornstarch 30%; milkfat 13%; cod liver oil 1%, wheat germ oil 1%, dried yeast 15%; salts 5% and cellophane 15%. In 1942 Hogan and Hamilton reported that guinea pigs and rabbits grew at the normal rate on simplified rations which contained dried yeast as the source of all water-soluble vitamins. When the water-soluble vitamins were supplied as pure compounds then available, the mortality was high and the rate of growth was subnormal.

Swaminathan('42) reported that "rabbits do not require an extraneous source of nicotinic acid and that they were able to synthesize it." His diet consisted of extracted oats 46%, sugar 10%, purified casein 10%, whole cow's milk 31% and salt mixture 3%. This basal diet was shown by analysis to contain an average of from 70 to 82 μg. of nicotinic acid per 100 gm. Five rabbits fed this diet gained an average of 235 gm. in 10 weeks. Five animals that were fed the diet plus 3 mg. of nicotinic acid gained an average of only 175 gm. in a similar period.

Under our experimental conditions reported below nearly normal growth has been maintained in rabbits for a limited period, at least, on our purified diet supplemented only with pure vitamin preparations. Rabbits fed this purified ration require the addition of niacin (nicotinic acid) for satisfactory growth.

EXPERIMENTAL

White rabbits from a strain raised at the National Institute of Health were used throughout these studies. The rabbits were housed in individual metal cages with half-inch wire mesh false bottoms. It was necessary to teach the animals to eat the purified diets. This was accomplished by including 25-gm. rabbit pellets (the stock ration fed the breeding colony) with 50 gm. of the experimental diet for 2 days and by adding about 5 gm. of the diet to each 100 ml. of their water supply for 1 day at the beginning of the experiment. After this procedure, the rabbits with few exceptions ate the purified diets readily.

The components of the diets used in these studies are shown in table 1.

TABLE 1

Composition of purified diets.

	DIET NUMBER			
	672	673	674	675
	Grams per 100 gm. of diet			
Casein [1]	20	20	20	20
Wesson oil	5	5	5	5
Sucrose	55.4	55.4	55.4	55.4
Cellophane [2]	15.6	15.6	15.6	15.6
Salt mixture [3]	4	4	4	4
Supplement [4]				
	Milligrams per 100 gm. of diet			
Carotene [5]	87	87	261	261
Drisdol [6]	20	20	60	60
a-Tocopherol	7.5	7.5	22.5	22.5
Choline	200	200	600	600
Niacin (nicotinic acid)	20	..	60	..
Inositol	10	10	30	30
	Micrograms per 100 gm. of diet			
2-Methyl-4-naphthoquinone	75	75	225	225
Pyridoxin	700	700	2100	2100
Thiamine	700	700	2100	2100
Riboflavin	700	700	2100	2100
Calcium pantothenate	1500	1500	4500	4500

[1] Casein was leached with 3 changes of 2% acetic acid, dried over heat, ground and washed with 3 changes of 70% alcohol.

[2] 780 gm. of sucrose was dissolved in water to make a thick syrup then thoroughly mixed with 220 gm. of PT glycerine softened cellophane flakes (E.I. duPont de Nemours and Co.), dried over a low flame and finely ground in a burr mill.

[3] The salt mixture was prepared by the method described by Osborne and Mendel (J. Biol. Chem., vol. 37, p. 572 ('19)) except that the following changes were made: NaF was reduced to 1% of the original value and 0.2 gm. $CuSO_4$ was added.

[4] The water-soluble vitamins were dissolved in water and mixed thoroughly with the casein; the oil-soluble vitamins were then mixed with the Wesson oil and mixed with the casein water-soluble vitamin mixture. The sucrose-cellophane mixture was mixed with the salt mixture then mixed thoroughly with the casein-vitamin mixture.

[5] Carotene-in-oil 7500 U.S.P. units per gram.

[6] Drisdol contained D_2 40,000 units per ml. in propylene glycole.

Experiment 1. Six rabbits, approximately 10 weeks old, weighing from 1600 to 1850 gm. were placed on diet 672 and observed for 21 weeks. The average weight gain of these rabbits was rapid for 16 weeks when an average gain of 1173 gm. per rabbit was reached. Records kept by the breeders of the stock colony of rabbits used in these experiments show that the average gain in weight of the 10-week-old rabbits fed the stock ration is 1100 to 1300 gm. in 15 weeks; following this period the rate of weight gain is less. Little change in the average weight was noted during the remaining 5 weeks of observation (fig. 1). Considerable variation in the weight of individual rabbits was noted but this might be expected since the animals were not litter mates and were fed ad libitum.

Experiment 2. After it had been determined that rabbits would survive and grow well when fed this purified diet, studies were begun to determine if niacin was necessary for survival and growth. Eleven pairs of litter mates (nine pairs of males and two of females) were placed on study as they became available. The weights of the animals were from 1100 to 1850 gm. Litter mates of as near equal weight as practicable were used. The greatest difference was 230 gm. All of the rabbits were fed ad libitum. One animal of each litter received the niacin deficient diet 673 while its mate was fed the control diet 672. Assay of the diets for niacin by a slight modification of the method of Snell and Wright ('41) revealed that the control diet 672 contained 0.21 mg. of niacin per gram whereas the quantity in diet 673 was too low to be determined. These rabbits were observed for 19 weeks. Seven of the eleven rabbits fed the niacin deficient diet died before the end of the period of observation. When one of the animals died, its corresponding litter mate was withdrawn from the test. Two of the rabbits that were fed the niacin deficient diet gained 330 and 340 gm., respectively. Even with these gains the animals on the deficient diet maintained an average weight that was less than the starting weight. The litter mates that received the control diet grew at an almost normal rate (fig. 2).

The niacin deficient animals showed marked emaciation and the majority had diarrhea, frequently bloody, shortly before death. Careful examination of the mouth failed to reveal any significant changes in the buccal mucosa. At autopsy, the only consistent findings were liquefaction of the fecal matter and gaseous distension of the intestines. Occasionally ascites was present. It was felt that there was not enough evidence to regard these findings as pathognomonic of niacin deficincy.

Experiment 3. Six pairs of litter mates were placed on paired feeding. The diets employed were control diet 674 and niacin deficient diet 675.

These diets contained three times the amount of vitamins in diets 672 and 673 to assure protection against multiple vitamin deficiencies developing in animals fed restricted amounts of diet. After the first 2 or 3 weeks the animal on the niacin deficient diet ate the least and therefore controlled the amount of ration fed its litter mate. Each pair of rabbits was observed until one of the animals died at which time its litter mate was sacrificed. Three rabbits on the control diet 674 and three fed the niacin deficient ration 675 died before their respective litter mates. The length of time on the rations varied from 38 to 149 days.

The ration consumed varied between 29.1 and 42.6 gm. per day by the rabbits of the various pairs. The diet was calculated to contain 3.4 cal. per gm. Each rabbit, therefore, consumed from 99 to 144 cal. per day, or from 79 to 104 cal. per kilogram of body weight. Except for one pair of rabbits, nos. 139 and 140, the difference in weight change of the litter mates while on the test was comparatively small. Five of the rabbits receiving the niacin deficient diet showed an average loss of 110 gm. from the starting weight and their litter mates receiving the control diet lost an average of 150 gm. Rabbit no. 139 fed the deficient diet lost 560 gm. during the 149 days of the experiment while its litter mate on the control diet gained 590 gm.

The antemortem symptoms and postmortem findings among the rabbits dying in this experiment (diarrhea just before death and liquefaction of fecal matter and increased gas in the intestines at postmortem examination) were similar to those shown by the niacin deficient rabbits in the preceding experiment. These findings were shown by the rabbits that had received the control diet as well as those that received the niacin deficient diet. It is possible that the signs and symptoms shown by these rabbits, as well as those fed the niacin deficient ration in the previous experiment, were due to slow starvation brought about by anorexia when niacin was excluded from the diet.

Histologic examination of the tissues from rabbits in these two experiments were made.[1] The tissues examined were the heart, lungs, liver, pancreas, spleen, adrenals, kidneys, small and large intestines, hamstring muscles, sciatic nerve, lumbar, dorsal and cervical vertebrae (including muscle, bone, bone marrow and cord) and four levels of the brain.

Tissues were fixed in formalin, embedded in paraffin and stained with eosinated polychrome methylene blue and with van Gieson's connective tissue stain. Weigert-Pal myelin stains of brain, spinal cord, and sciatic nerve were prepared. Marchi preparations or Sudan IV stains of frozen sections were made in a few cases.

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[1] The histological examinations were made by Surgeon (R) G. L. Fite and Passed Assistant Surgeon K. M. Endicott, Division of Pathology, National Institute of Health.

There were no consistent lesions which could be attributed to niacin deficiency. Two of the thirteen rabbits examined receiving no niacin showed superficial degeneration of the mucosa of the colon consisting of cytoplasmic oxyphilia and nuclear karyorrhexis. Both rabbits (nos. 95 and 151) had diarrhea. Three other niacin deficient rabbits and one control rabbit had diarrhea but showed no lesions in the colon. Atrophy and necrosis of skeletal muscle, atrophy of fat, and hypocellularity of bone marrow were noted in several rabbits receiving niacin in the diet as well as in several of those receiving no niacin.

Assays for niacin were made on representative samples of liver, kidney, and muscles of the hind leg of nine of the eleven pairs of rabbits that were fed ad libitum and of the six pairs of animals that were pair fed. By referring to table 2 it will be noted that there was no significant difference of niacin content of the respective tissues of the litter mates that had been on tests less than 60 days. If all rabbits are considered, the difference in the niacin content of the muscles of the deficient and the control animals is of borderline significance. If, however, only the rabbits that were on diet more than 15 weeks are considered there is a definite diminution in the niacin content of the muscles of the deficient animals as compared to their control litter mates.

Experiment 4. The next study was to determine whether reversing the diets after the rabbits had been fed the niacin deficient or the control ration would effect their growth rate. One of each of four pairs of litter mates was fed the niacin deficient diet 673 and its mate the control diet 672. The diets were reversed at the end of 7 weeks and the rabbits that had received the niacin deficient diet were changed to the control diet, and those that had received the control diets were given the niacin deficient diets. At the time the groups were reversed the control rabbits had gained 560, 670, 880 and 330 gm., respectively, while those on the niacin deficient diet had a weight change of +10, —20, —160, and —260 gm. Following the reversal of the diets the rabbits that had been fed the control diet 672 but that now received the deficient diet usually gained less rapidly or showed a loss of weight. At the end of the next 7–week period these rabbits had a weight change of +120, +320, —60, and +10 gm., respectively, while those that had been fed the niacin deficient diet 673 but now received the control diet gained 690, 940, 790, and 800 gm. (fig. 3). Thus it is shown that if rabbits are fed a purified diet with adequate supplements of the more common pure vitamins their growth is rapid whereas if the same diet is employed but without the addition of niacin as the only difference, the weight increase is small or loss of weight may occur.

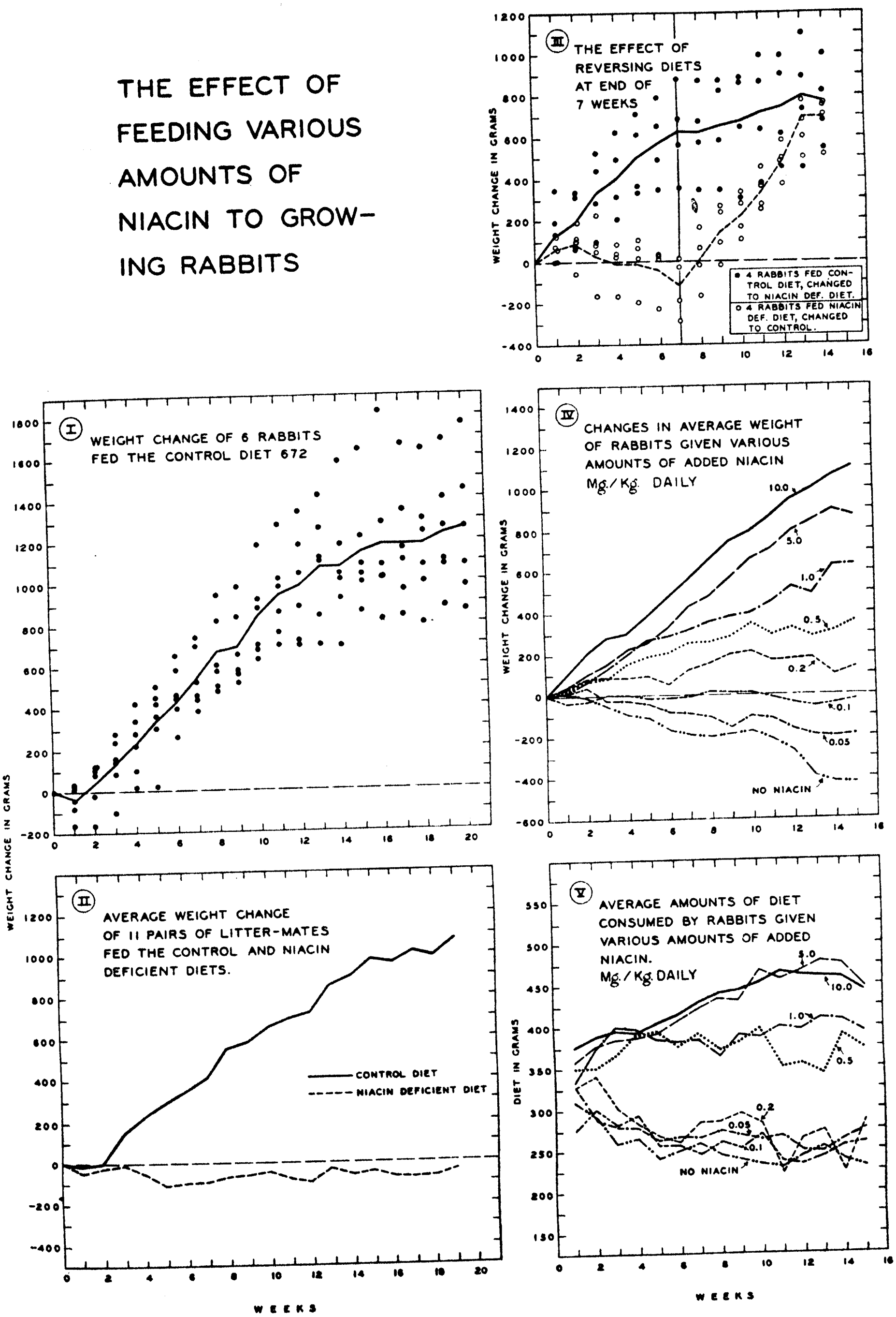
Experiment 5. An experiment was next set up to determine the niacin requirement of rabbits when fed the purified diet.

Ten groups of eight rabbits each, without regard to litter mates, were fed the niacin deficient diet 673. Supplements of niacin equal to 0.0, 0.05, 0.1, 0.2, 0.5, 1.0, 2.5, 5.0, 7.5, and 10.0 mg. per kilogram of body weight daily were added to the diets of the rabbits in the respective

TABLE 2

Results of niacin assays of tissues of litter mate rabbits that had been fed niacin deficient and control diets.

PAIR NO.	NO. DAYS ON DIET	TISSUES ASSAYED	NIACIN CONTENT IN RABBITS FED		PAIR NO.	NO. DAYS ON DIET	TISSUES ASSAYED	NIACIN CONTENT IN RABBITS FED	
			Niacin-deficient diet	Control diet				Niacin-deficient diet	Control diet
Ad libitum feeding of niacin-deficient diet 673 and control diet 672									
			µg./gm. of tissue					µg./gm. of tissue	
89	180	Liver	187	150	151	44	Liver	120	175
and		Kidney	87	94	and		Kidney	78	100
90	180	Muscle	90	141	152	44	Muscle	115	130
93	259	Liver	135	242	153	47	Liver	150	140
and		Kidney	68	100	and		Kidney	105	110
94	259	Muscle	90	135	154	47	Muscle	105	125
95	195	Liver	175	190	155	156	Liver	125	170
and		Kidney	65	89	and		Kidney	110	112
96	195	Muscle	71	150	156	156	Muscle	104	135
99	107	Liver	80	160	157	51	Liver	100	120
and		Kidney	82	110	and		Kidney	100	110
100	107	Muscle	55	150	158	51	Muscle	120	120
149	175	Liver	198	248					
and		Kidney	112	83					
150	175	Muscle	67	135					
Paired feeding of niacin-deficient diet 675 and control diet 674									
137	94	Liver	140	144	143	38	Liver	120	110
and		Kidney	86	100	and		Kidney	95	80
138	94	Muscle	93	139	144	38	Muscle	100	...
139	147	Liver	150	167	145	119	Liver	166	166
and		Kidney	108	...	and		Kidney	73	144
140	147	Muscle	73	155	146	119	Muscle	80	...
141	110	Liver	165	195	147	40	Liver	110	140
and		Kidney	110	110	and		Kidney	75	110
142	110	Muscle	136	106	148	40	Muscle	120	110



Figures 1 to 5

groups three times each week. The animals were observed for 15 weeks. The weight of the rabbits and the ration consumed was recorded each week.

Considerable variations in the weight change of the individual rabbits of each group were observed. One of the eight rabbits that were supplemented with 1.0 mg. of niacin per kilogram of body weight daily, two of those that received 2.5 mg., three that were fed 5.0 mg., four that ate 7.5 mg., and seven of the eight animals that received the equivalent of 10.0 mg., grew at a rate comparable with the average rabbit that had received the control diet 672. Deaths occurred in each of the groups that were supplemented with 1.0 mg. of niacin/kg. of body weight or less. Each of these animals failed to grow and had diarrhea shortly before death. One rabbit in each group receiving 1.0, 2.5, and 5.0 mg. niacin, respectively, met with accidents and was discarded.

The average weight of the rabbits in each group increased with each added amount of niacin until the level of 10.0 mg./kg. of body weight daily was reached (fig. 4). This observation suggests that it may be possible to use the rabbit as a biological assay animal for niacin if significant numbers are employed.

The average weekly consumption of food by the groups that received 0, 0.05, 0.1 and 0.2 mg., respectively, of niacin per kilogram of body weight daily was less as the experiment progressed than at the beginning of the test. When 0.5 or 1.0 mg./kg. of niacin was given, the amount of food eaten was about the same throughout the period of observation. But when 5.0 or 10.0 mg./kg. was supplied, the average amount of diet consumed increased as the test progressed (fig. 5).

CONCLUSIONS

1. Young rabbits fed a purified ration supplemented with pure vitamin preparations grew at a nearly normal rate.
2. Rabbits fed this purified diet require niacin for survival and adequate growth.
3. Symptoms shown by rabbits receiving the niacin deficient diet are loss of weight and usually severe diarrhea 24 to 48 hours before death.
4. Rabbits fed the diet containing adequate amount of niacin but pair-fed with litter mates receiving the niacin deficient ration died as early as the deficient animals and showed simliar symptoms.
5. Rabbits fed the niacin deficient diet for 3 months or longer showed significantly less niacin in the voluntary muscles than the litter mates given niacin.

6. Under the conditions of our experiments rabbits fed the purified diet showed considerable individual variation in growth response to added niacin. Maximum average growth of groups of eight rabbits was not reached until an amount of niacin equal to 10 mg./kg. of body weight daily was added.

7. Groups of eight rabbits fed the niacin deficient diet supplemented with graduated amounts of niacin gave average growth responses directly related to the amount of niacin fed.

8. The only symptom which could be attributed to niacin deficiency was anorexia; the other symptoms manifested by the deficient rabbits may have been due to slow starvation.

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EFFECT OF SUNSHINE UPON THE ASCORBIC ACID AND RIBOFLAVIN CONTENT OF MILK[1]

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ONE FIGURE

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Milk is very generally considered a rich natural source of riboflavin for the human dietary. Milk also contains appreciable amounts of ascorbic acid. In an extensive review of the literature Booher, Hartzler and Hewston ('42) found that the ascorbic acid content of whole milk varied from 0.07 mg. to 29.2 mg. per 100 gm. and riboflavin varied from 60 μg. to 342 μg. per 100 gm., apparently depending upon the part of the world in which the milk was produced and upon the breed, feed and physical condition of the cow. However, data concerning the amount of ascorbic acid and riboflavin in milk when drawn from the cow do not supply the ultimate consumer with information regarding the amounts of these vitamins he obtains in the milk that he consumes. The influence of factors such as pasteurization, bottling, lapse of time, storage by the producer or dispenser, storage on the customer's doorstep and in the home refrigerator, operates between the time the milk is drawn from the cow and its ultimate use. Holmes and Holmes ('43) reported that milk of quite uniform riboflavin content could be produced by a sizeable mixed herd of cows maintained under standardized conditions, and Holmes ('44) found very little destruction of riboflavin when milk was pasteurized by the holding or flash process. On the other hand, Williams and Cheldelin ('42), Ziegler ('44), Peterson, Haig, and Shaw ('44) and Stamberg and Theophilus ('44) have submitted conclusive evidence that milk may lose a considerable portion of its riboflavin while it is exposed to sunshine on the consumer's doorstep. Unfortunately these investigators did not report information concerning the amount of the sun's energy that fell upon the milk bottles or the temperature of the milk during its exposure to sunshine. The study reported in this paper was conducted to accumulate data concerning these factors.

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[1] Contribution no. 542 from the Massachusetts Agricultural Experiment Station.

The milk used in this study was supplied by the Dairy Manufacturing Laboratory where it was pasteurized at about 145°F. for 30 minutes. As soon as the milk was cooled it was bottled in one-half pint flint glass bottles and exposed to sunshine. The bottles, which varied from day to day, were assumed to be typical of those used by commercial dairymen. In order to provide uniform exposure conditions, the milk was placed on the flat roof of a four-story building 67 feet above the ground and thus all shadows were eliminated. The sample bottles of milk were arranged on a large sheet of brown manila paper so as to minimize any absorption or reflection of sunshine in the area surrounding the samples of milk.

The temperature of the milk when it was exposed to the action of sunlight varied from day to day but it was hoped that it was similar to that of commercial milk when delivered to the individual customer by the milkman. It was recognized that the effect of exposing milk to sunshine might be influenced somewhat by the temperature of the milk during exposure. Accordingly a record was made of the temperature of the milk and of the air surrounding the milk bottles at the beginning and end of the exposure periods. A thermometer was placed in the milk with the bulb at the intersection of the horizontal and vertical centers of the bottle. Inasmuch as the amount of sunshine which falls on milk bottles remaining on doorsteps varies with meteorological conditions, an attempt was made to expose milk on very cloudy days when there was little or no sunshine, on days of intense sunshine and on days when the sunshine was intermittent. There were two 30-minute or two 60-minute exposure periods for each bottle of milk and the second exposure followed immediately after the first.

The intensity of the sunshine to which the milk was exposed was measured continuously by a pyrheliometer equipped with an automatic recording device. The pyrheliometer was placed near the bottles of milk which were being exposed.

ASSAY METHODS

The effect of exposure of milk to sunshine was judged by the ascorbic acid and riboflavin content of the milk just previous to and immediately following exposure. Assays of the milk were commenced as soon as its exposure to sunshine was completed. The ascorbic acid content of the milk was determined by the method employed in previous studies, namely, that reported by Holmes, Tripp, Woelffer, and Satterfield ('39). The riboflavin assay procedure which was employed has been previously described by Holmes, Jones, Wertz, and Kuzmeski

('43) except that potassium permanganate and hydrogen peroxide solutions were used instead of stannous chloride and sodium hyposulphite to discharge any possible extraneous color in the milk.

RESULTS

Temperature. Detailed data concerning the conditions of the exposure of the milk to sunshine are reported in table 1. It will be noted that on different days the temperature of the air surrounding the milk at the start of the first exposure period varied from 18.0° to 30.0°C. At the end of the first period the temperature varied from 18.0° to 33.0°C., and at the end of the second exposure period, which immediately followed, the temperature range was from 20.0° to 34.5°C. The temperature of the 13 samples of milk when first exposed to sunshine varied from 8.0° to 11.5°. At the end of the first period the temperature ranged from 15.0° to 30.5° and by the end of the second exposure period the temperature of the milk varied from 18.0° to 38.5°C. It is obvious from these data that the different samples of milk were exposed to sunshine

TABLE 1

Data concerning exposure of milk to sunshine.

SAMPLE	TEMPERATURE (DEGREES C)						INTENSITY OF SUNSHINE (GM. CAL. PER SQ. CM. PER MINUTE)					
	First period				Second period		First period			Second period		
	Start		End[1]		End		Minimum	Maximum	Average	Minimum	Maximum	Average
	Air	Milk	Air	Milk	Air	Milk						
1	18.0	9.5	18.0	15.0	20.0	18.0	.00	.13	.08	.13	.23	.14
2	20.0	9.0	22.0	18.5	24.0	22.0	.13	.36	.29	.35	.46	.39
3	18.5	11.5	21.0	19.0	20.0	23.0	.20	.58	.33	.58	1.14	.90
4	26.5	9.0	28.5	19.0	31.0	26.5	.59	.76	.67	.59	.87	.76
5	24.5	8.0	25.5	18.0	27.0	24.0	.43	.94	.73	.86	1.09	.98
6	23.5	9.5	26.0	22.5	29.0	27.5	.35	.42	.37	.40	.64	.46
7	22.5	8.0	25.0	18.0	26.0	25.0	.81	.99	.93	.84	1.06	.88
8	24.0	10.0	25.5	21.0	25.0	24.5	.94	1.09	.99	1.07	1.22	1.13
9	27.0	11.5	27.0	26.0	33.0	33.5	.28	.86	.56	.50	1.19	.93
10	20.5	10.5	22.0	20.0	26.5	27.5	.26	.99	.58	.45	1.52	1.28
11	30.0	10.5	33.0	30.5	34.5	38.5	.76	.96	.88	.96	1.09	1.06
12	23.5	7.0	28.0	23.5	28.5	31.0	.85	1.22	1.00	.99	1.34	1.24
13	27.5	9.0	28.5	27.5	30.0	32.0	.99	1.19	1.13	1.19	1.39	1.28

[1] Readings for end of 1st period and start of 2nd period identical.

Samples 4, 5, 7 and 8 were exposed for two 30-minuute periods.

All the other samples were exposed for two 60-minute periods.

under a wide variety of conditions. In general the temperature of the milk increased during the first hour to within 3° or 4° of the temperature of the air surrounding it. By the end of the second hour the temperature had increased to about that of the surrounding air and in some instances exceeded it depending upon the intensity of the sunshine, the wind velocity and temporary cloud effects. Also, probably the milk bottles tended to develop the greenhouse effect, namely, as in the case of greenhouses the amount of heat from the sun that entered the bottles exceeded that which left them.

Intensity of sunshine. In order that the variability of the intensity of the sunshine to which the milk was exposed may be visualized, data are recorded in table 1 concerning the minimum, maximum and average gram calories per square centimeter per minute for each sample of milk during the first and second exposure periods. In an attempt to obtain as much variation of sunshine intensity as possible some samples (4, 5, 7 and 8) were exposed to sunshine for two 30-minute periods, and the remaining samples were exposed for two 60-minute periods. The total amount of sunshine that fell upon the bottles of milk was computed by multiplying the average gm. cal. per sq. cm. for the period by the number of minutes of exposure. The sunshine varied during the exposure of the different samples of milk from a total of 4.8 gm. cal. per sq. cm. for the initial period for sample 1 to 144.6 gm. cal. per sq. cm. for the combined periods for sample 13. It was relatively easy to obtain a total of less than 70 gm. cal. per sq. cm. during a 1- or 2-hour period, but it was not convenient to obtain many exposures of milk at higher sun intensities during the experimental period and it did not seem feasible to extend the period since under practical conditions probably milk is not allowed to stand on the customer's doorstep for more than 2 hours of sunshine.

Effect of sunshine on the reduced ascorbic acid in milk. The ascorbic acid content of the samples of milk at the beginning of the exposure period varied from 13.6 to 16.8 mg. per liter. The value 5.2 mg. per liter for sample 11 should not be considered normal because the sample stood for about 4 hours exposed to light in the laboratory on a very warm day before it was assayed. The amount of ascorbic acid in the milk at the end of the first period varied from 0.0 to 4.8 mg. per liter. At the end of the second exposure period only two samples contained any reduced ascorbic acid. It is apparent from these data that reduced ascorbic acid in milk is very rapidly oxidized when the milk is exposed to sunshine. In fact, samples 4, 5, 7 and 8 showed no ascorbic acid after 30 minutes exposure to bright sunshine.

This observation is in agreement with those of Kon and Watson ('36) who found that milk exposed to June skyshine lost all its reduced ascorbic acid within the first hour of exposure. They concluded that in general a pint bottle of milk exposed under practical conditions for one-half hour in the sun loses fully one-half of its original antiscorbutic properties. The rapid loss of ascorbic acid noted in this study when milk was exposed to sunshine is in accord with earlier observations by Krauss ('40) who reported that pasteurized milk kept in a clear bottle at room temperature lost practically all of its vitamin C within 6 hours. Diemair and Fresenius ('40) were much impressed by the possible rapid destruction of vitamin C, for they reported the loss of vitamin C by exposure of milk to light while carrying out analyses Concerning the cause of the loss of reduced ascorbic acid from milk exposed to light or sunshine Buruiana ('37) stated that the action of sunlight on milk produces oxidation of unsaturated fat, and oxidation by catalytic dehydrogenation of the ascorbic acid present in the milk; and Hand, Guthrie and Sharp ('38) have stated that lactoflavin is the sole agent in milk responsible for the sensitivity of ascorbic acid to light. In a study of the pigments, vitamins and enzymes of milk in relation to changes in flavor and nutritive value, Hand and Sharp ('41) found that "riboflavin, the fluorescent green coloring matter in whey, is responsible for the oxidation of vitamin C in light."

However, these data do not prove that such milk is devoid of biological vitamin C value. Washburn and Krauss ('38) reported that milk stored 6 hours and 24 hours in ordinary milk bottles in a cooler with uncertain refrigeration showed, respectively, a 12.9% and 29.4% loss of ascorbic acid, measured by titration; but in a corresponding biological test with guinea pigs, in which light and temperature were not controlled during the storage of the milk, they did not find a corresponding decrease in the biological activity of the stored milk. Hand ('43) reported that light causes an oxidation of the reduced ascorbic acid in milk to dehydroascorbic acid which has biological value but the amount present in milk at any time depends on the rate of formation and the rate of destruction of dehydroascorbic acid. Kon and Watson ('36) found that exposure of milk to light for ½ hour destroyed all the reduced ascorbic acid and in the absence of light the larger portion of the dehydroascorbic acid disappeared within the succeeding 5 hours.

It is quite evident from the cited data and those assembled in this study that milk exposed to sunshine on the consumers' doorstep may lose much of its value as a source of vitamin C.

Effect of sunshine on the riboflavin in milk. It was anticipated that sunshine would have a significant destructive effect on the riboflavin content of milk, for Hogan and Hunter ('28) found that riboflavin was destroyed by exposure to ultra-violet rays of a quartz mercury vapor lamp for 10 hours and György ('35) reported that lactoflavin was destroyed by visible light. However, Fuhr, Dornbush and Peterson ('43) reported that experimental and market milk irradiated with ultra-violet light to produce 400 U.S.P. units of vitamin D per quart were not reduced in riboflavin content.

TABLE 2

Destructive effect of sunshine on the ascorbic acid and riboflavin content of milk. (mg./liter)

SAMPLE	ASCORBIC ACID			RIBOFLAVIN		
	Start	End 1st period	End 2nd period	Start	End 1st period	End 2nd period
1	16.2	4.8	0.8	1.88	1.69	1.41
2	15.7	0.4	0.0	1.53	1.11	.67
3	14.0	0.0	0.0	1.74	1.44	.66
4	14.9	0.2	0.0	1.89	1.52	.86
5	16.0	0.0	0.0	1.66	1.22	.86
6	16.3	0.8	0.0	1.70	1.28	.58
7	15.5	trace	0.0	1.69	1.18	.62
8	13.6	0.0	0.0	1.90	1.28	.69
9	16.4	0.4	0.0	1.72	.99	.41
10	14.4	..	0.4	1.57	1.14	.43
11	5.2[1]	0.0	0.0	1.57	.24	.25
12	16.8	0.0	0.0	1.48	.60	.30
13	15.9	0.0	0.0	1.78	.64	.36

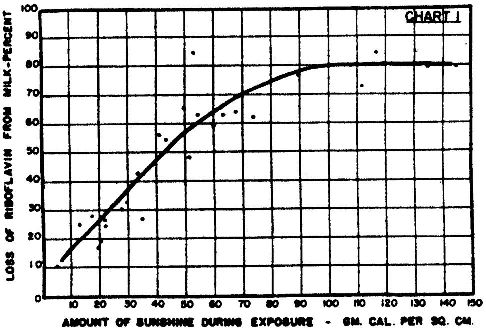
[1] Sample 11 stood several hours in a warm room before assay.

Samples 4, 5, 7 and 8 were exposed for two 30-minute periods.

All other samples were exposed for two 60-minute periods.

The amount of riboflavin (table 2) in the milk at the beginning of exposure to sunshine varied from 1.48 mg. to 1.90 mg. per liter. At the end of the first period of exposure it varied from 0.24 mg. to 1.69 mg. and at the end of the second period of exposure the riboflavin content of the milk varied from 0.25 mg. to 1.41 mg. per liter. The smallest destruction of riboflavin, 10.1%, occurred during the first period of exposure of sample 1 on a rainy day, when the temperature of the milk varied from 9.5° to 18.0°C. The largest destruction, 84.5% for sample 11, occurred on a very warm day when the temperature of the milk was 38.5°C. at the end of the second exposure period. The data accumulated in this study indicate that the temperature of the milk influences the amount of destruction of riboflavin. This observation is in accord with those of Williams and Cheldelin ('42) who reported that increase in temperature accelerated the destruction of riboflavin, that the destruction of riboflavin in liquid foods proceeds at a rapid rate and that as much as 26% of the riboflavin in milk was destroyed by light in a 5-minute exposure at 100°C. Ziegler ('44) also found that the destruction of riboflavin in milk increases with temperature.

In order to facilitate comparing the effect of increasing the sun's intensity with the loss of riboflavin, the data concerning these factors are reported in chart 1. The points on the chart represent data for the intensity of the sunshine, the temperature of the milk, and the loss of riboflavin during the various exposure periods. The curve was drawn by inspection to represent the general average of the relation between the intensity of the sunshine and the riboflavin loss irrespective of the temperature of the milk. Inspection of the chart will reveal that in general as the intensity of the sunshine to which the milk was exposed increased, the riboflavin destruction increased quite consistently until 60–70% of the riboflavin was destroyed when the milk was exposed to 50–70 gm. cal. per sq. cm. There was a further, though very slow, increase in the destruction of riboflavin as the intensity of the sunshine increased from 70–140 gm. cal. per sq. cm. It is interesting to compare these findings with those of other investigators. Peterson, Haig and Shaw ('44) obtained as much as 48% destruction of riboflavin when fresh milk was exposed in pint bottles from mid-morning to mid-afternoon to direct sunlight on an open porch at temperatures of 60°F. to 72°F. Ziegler ('44) exposed several types of milk, 20 hours after bottling in quart bottles, to direct mid-morning spring sunshine in the open air at atmospheric temperatures between 16.7° to 20.6°. His pasteurized milk lost 54% of its riboflavin during 2 hours exposure. Stamberg and



Theophilus ('44) reported as much as 80% loss of riboflavin from milk exposed in clear glass quart bottles to March sunlight, 45°F., for 6 hours. When exposed for 2 hours the loss was 48.3%. However, such a comparison should include allowance for the difference in the surface area of the milk in half-pint, pint and quart bottles.

SUMMARY

A study has been made of the effect upon the ascorbic acid and riboflavin of exposing milk in commercial one-half pint bottles to the action of sunshine. The intensity of the sunshine was measured with a pyrheliometer equipped with an automatic recording device. The milk was exposed for two 30- or two 60-minute periods. The "sunshine" varied from a total of 4.8 gm. cal. per sq. cm. on a rainy day to 144.6 gm. cal. per sq. cm. on a bright day. The temperature of the milk varied from day to day depending upon velocity of the wind, greenhouse effect of the milk bottles and intensity of the sunshine.

The destruction of reduced ascorbic acid was very rapid, for little, if any, was present after 30 minutes exposure. The riboflavin disappeared more slowly than the ascorbic acid. There was a 10% loss during 60 minutes exposure on a rainy day and about 85% loss during exposure to bright sunshine for 120 minutes. In general the destruction of riboflavin increased fairly consistently with the increase in intensity of the sunshine until 60–70% of the riboflavin was destroyed when the milk was exposed to a total of 50–70 gm. cal. per sq. cm. Increasing the sunshine from a total of 70 to 140 gm. cal. per sq. cm. caused a slow increase in the destruction of riboflavin.

These data show that milk allowed to stand for more than a short period on the consumer's doorstep exposed to strong light or sunshine is likely to lose a large amount of its ascorbic acid and riboflavin.

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CITRATE METABOLISM OF PRESCHOOL CHILDREN

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The data presented here are part of a larger study on the influence of changes in the ascorbic acid and citrate content of the diet on the ascorbic acid, citric acid, calcium, phosphorus, and nitrogen metabolism of preschool children. This report presents data on the effect of supplements of ascorbic acid, potassium citrate, and orange juice on the excretion of citric acid by four children studied during 1941–42. The effect of these supplements on ascorbic acid excretion has been reported by Meyer and Hathaway ('44), and their effect on calcium, phosphorus, and nitrogen metabolism will be reported later.

EXPERIMENTAL

Plan of the experiment

The general plan of the complete experiment, including a description of all subjects and of the diets used, has been reported in detail by Meyer ('43) and summarized by Meyer and Hathaway ('44). The subjects for the citrate study included a boy, E, and three girls, F, G, and H. (Their respective ages and weights at the beginning of the study were 55, 49, 40 and 38 months, and 34, 40, 32, and 29 pounds.) They lived in the college laboratory-apartment for 5 months and were maintained on a basal diet adequate in all nutrients except ascorbic acid. Citric acid determinations were made for sixteen 7-day periods (periods 5 through 20). Milk and all supplements were given at the same level to all subjects, but somewhat less of the basal foods was consumed by H throughout the study, and by G during the last 5 weeks. Ry-Krisp or Toasted Wheat Wafers were allowed ad libitum. Supplements of crystalline ascorbic acid, potassium citrate, and orange juice were given as indicated in table 1. The 3.38 gm. of potassium citrate corresponds roughly to the amount of citric acid and its salts calculated to be present in 200 ml. of orange juice and to be associated with 100 mg. of ascorbic acid from this source. The amount of orange juice actually given was determined daily as that amount which would furnish 110 mg. of ascorbic acid. Ten mg. of the ascorbic acid of the orange juice replaced an equal amount of crystalline ascorbic acid included in the basal diet in all other experimental periods; the accompanying increase in citric acid and its salts was slight.

TABLE 1

Average daily intake and urinary excretion of citrates in four preschool children.

SUBJECT		E		F		G		H	
Diet	Period	Intake	Urinary Excretion	Intake	Urinary Excretion	Intake	Urinary Excretion	Intake	Urinary Excretion
		mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
I	7	1390	55	1390	260	1390	66	1312	81
	8	1337	56	1334	236	1334	105	1256	79
	9	1435	53	1432	244	1432	130	1339	119
	Av.	1387	55	1385	247	1385	100	1302	93
II	5	1385	63	1387	231	1387	109	1294	87
	6	1329	76	1329	230	1331	70	1239	59
	10	1415	66	1412	348	1411	74	1330	104
	11	1451	95	1448	250	1444	94	1367	77
	12	1535	62	1532	261	1528	84	1445	123
	Av.	1423	72	1422	264	1420	86	1335	90
III	13	3496	199	3493	341	3485	221	3399	210
	14	3511	225	3509	379	3498	237	3431	300
	15	3570	235	3567	416	3553	272	3486	268
	Av.	3526	220	3523	379	3512	243	3439	259
IV	16	3600	236	3598	423	3495	275	3504	263
	17	3568	236	3568	472	3464	292	3474	239
	18	3690	283	3687	514	3577	278	3586	186
	Av.	3619	252	3618	470	3512	282	3521	229
V	16	3600	236	3598	423	3495	275	3504	263
	20	3599	140	3596	357	3498	236	3498	151
	Av.	3700	157	3697	390	3598	238	3600	153

I. Basal diet (containing 10 mg. crystalline ascorbic acid).
II. Basal diet plus 100 mg. ascorbic acid.
III. Basal diet plus 3.38 gm. potassium citrate (citrate content equivalent to 2.19 gm. citric acid).
IV. Basal diet plus 100 mg. ascorbic acid plus 3.38 gm. potassium citrate.
V. Basal diet without crystalline ascorbic acid, but plus orange juice containing 110 mg. ascorbic acid and an average citrate content equivalent to 2.29 gm. citric acid.

Collection and preparation of samples for analyses

Urines. Half of each urine specimen was preserved with thymol for pH determinations and for citric acid and mineral analyses. At the end of 24 hours the pH of the sample for that period was determined; then sufficient hydrochloric acid was added to make a 2% solution, and two-fifths of the sample was saved for the weekly composite specimen later used for citric acid and mineral analyses. All samples were stored under refrigeration.

Foods. At each meal aliquots were taken of all basal foods except milk. These were placed in Pyrex flameware saucepans and digested with 1:4 hydrochloric acid according to the method of Stearns ('28–'29). Composite samples for a weekly period were brought to volume in three 2000 ml. volumetric flasks, and aliquots were stored for analysis. The proteins of the food digests were removed by precipitation with trichloracetic acid at the time of analysis. Samples of Ry-Krisp and Toasted Wheat Wafers were partially digested with hydrochloric acid and then treated in the same manner as other food digests. Samples of the milk for each week[1] were also analyzed separately. The milk sera were prepared for analysis by the method of Lampitt and Rooke ('36).

Citric acid determinations

The Kuyper and Mattill ('33) modification of the pentabromacetone method was used for the citric acid determinations.

RESULTS AND DISCUSSION

Urinary excretion of citrates

The average daily values for the intake and urinary excretion of citrates (calculated as citric acid) by the four subjects are given by periods and by supplements in table 1. The intakes for all subjects were similar, but the excretion values for subject F were markedly higher than those for the other three children, regardless of supplement. The values for G and H were in the same range for diets I, II, and III, but those for H were lower on diets IV and V. The values for E were lower than those for G and H on diets I, II, and III, and between the G and H values on diets IV and V.

A comparison of the intakes and urinary excretions of citrates per kilogram of body weight was made. The average intakes per kilogram at the basal citrate level varied from 75 mg. (for F) to 96 mg. (for H); when potassium citrate was added to the basal diet, the intakes varied from 184 mg. to 244 mg. (for F and H, respectively). The basal citrate excretion values ranged from 3 to 9 mg./kg. for E, G, and H, but from

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[1] Specially prepared by Prof. E. S. Guthrie so that it retained its ascorbic acid content and fresh flavor for at least a week. See Sharp, Hand and Guthrie ('39).

13 to 19 mg. for F. When potassium citrate was added to the basal diet, the citrate excretions for E and G ranged from 12 to 18 mg./kg., for H from 13 to 22 mg., and for F from 18 to 26 mg. When orange juice was added to the basal diet the excretion values for all the children were lower than on the potassium citrate supplement, averaging 9 to 14 mg./kg. for E, G, and H, and 19 mg. for F. The excretion values for F were, with one exception (H on diet III), still markedly higher than those for the other children. It is interesting to note that on a per kilogram basis, subject F, who generally maintained the highest excretion values, consistently had the lowest citrate intake. This is in agreement with the observations of Östberg ('31), substantiated by those of other workers, that the citrate excreted is of endogenous origin, and is independent of the citrate intake.

The individual variations among our subjects on a standardized diet are not surprising. Meyer and Smith ('40) in a study on rats noted considerable variation in citric acid production within a group on a given diet. They also observed that the fluctuations for any one rat were within narrow limits, so that a distinct individuality was discernible.

The values for the urinary excretion of citrates observed with these children correspond to those cited in the literature. McClure and Sauer ('22) observed excretions of 64 to 254 mg. for children of this age range (3 to 5 years) on uncontrolled citrate intakes. Sherman, Mendel, and Smith ('36) reported excretion values for normal adults of 356 to 1180 mg., or 5 to 20 mg./kg. In our subjects the values per kilogram varied from 3 to 26 mg., but only 2 values (3% of the total) were more than 2 mg. outside the limits given for adults.

The effect of changes in the ascorbic acid content of the diet on the citrate excretion

Interest in the possible interrelationships of ascorbic acid and citric acid was aroused by the papers of Purinton and Schuck ('43a, '43b). From the data in table 1 the possible effect on citrate excretion of adding 100 mg. of ascorbic acid to the basal diet or to the basal diet supplemented with potassium citrate is observable. The average excretion values were higher in five and lower in three of the eight cases. Statistical treatment of the data, however, indicates that the differences noted were significant only for F on the higher level of citrate intake (odds greater than 30:1, using Student's "t" table). It is realized that statistical treatment has limited value when so few cases are compared, but it does confirm the conclusions drawn by inspection of the data, that the addition of ascorbic acid did not cause consistent increases in citrate excretion.

Comparison of the ascorbic acid "utilization"[2] values (Meyer and Hathaway, '44) with the citrate excretion values reveals that subject E showed the greatest increase in "utilization" of ascorbic acid at both levels of citrate intake, and showed no tendency to lowered citrate excretion. Subject H, who showed the least increase in "utilization" of ascorbic acid, showed no tendency to increased citrate excretion. Thus the results of this study do not appear to be in agreement with those of Purinton and Schuck ('43b) on human subjects. However, since our citrate values are averages rather than daily values, possibly there was an adjustment to the changed intake with time.

The effect of addition of potassium citrate to the diet on citrate excretion

The various supplements to the basal diet make possible a number of comparisons of the citrate excretion at different levels of citrate intake, e.g., the differences in excretion using potassium citrate as the source of added citrate (1) on a low ascorbic acid intake, (2) on a high ascorbic acid intake, and (3) ignoring the ascorbic acid intake.

An increase in citric acid excretion on the administration of potassium citrate was noted for all subjects; and, if the effect of the added citrate is considered rather than that of the total citrate, the increment of citric acid excreted was similar for all subjects. At the basal level of ascorbic acid intake, the addition of potassium citrate resulted in an increase in citrate excretion of 132 to 166 mg., (average excretion on diet I subtracted from that on diet III) or 6.2 to 7.8% of the increased intake. At the higher level of ascorbic acid intake this difference amounted to 139 to 206 mg., (average citrate excretion on diet II substracted from that on diet IV) or 6.4% to 9.4% of the added citrate. When the changes in ascorbic acid are ignored, and the changes in citrate level only are considered (average of all values for citrate excretion on diets I and II substracted from the average on diets III and IV) the range of values is even smaller, 153 to 167 mg., or 7.1 to 8.1%.

Schuck's studies ('34b) on the effect of sodium citrate on the citrate excretion in four adults may be compared with the present study on the affect of added potassium citrate. Sodium citrate equivalent to 12 gm. of citric acid was added to her basal diet. Of this, 856 to 1898 mg. were excreted, equivalent to 7.1 to 15.8% of the added intake. Subject S showed exceptionally high excretion except on the basal diet. If her values are omitted, the range of excretion with the sodium citrate was 7.1 to 9.1%, or is similar to the range found in the present study with potassium citrate.

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[2] The term "utilization" is used arbitrarily to refer to the difference between intake and excretion: corresponds to the term "retention" in the reports of Purinton and Schuck.

The effect of addition of citrus fruits to the diet on citrate excretion

At the higher level of ascorbic acid intake the addition of citrate as orange juice resulted in an increase in citrate excretion ranging from 63 to 126 mg. (average citrate excretion on diet II substracted from that on diet V) or 2.8 to 7.0% of the added intake. For each subject the increase in excretion was less when the citrate was added as orange juice than when it was added as potassium citrate.

In 6 subjects given 1000 ml. of orange juice daily, Schuck ('34a) found an excretion of 234 to 1294 mg., or 2.6 to 16.6% of the added orange juice. Again, if the values for subject S are omitted, the range was 234 to 443 mg., or 2.6 to 7.5%. Lanford ('42) reported that with the addition of 1800 ml. of grapefruit juice the range of "extra" citrate found in the urine of her subjects was 112 to 738 mg., or 0.67 to 3.42% of the increased intake. The range found in the present study when orange juice was added to the basal diet (2.8 to 7.0%) is similar to that found by Schuck ('34a).

Relationship of citrate excretion to acid-base balance

Changes in the ascorbic acid content of the diet made little or no change in the pH of the urine, and also caused little change in the citrate excretion in the four subjects of this study. The addition of potassium citrate to the diet, however, increased the average urinary pH by 0.9 to 1.4 units, from averages of 5.5 to 5.9, to from 6.8 to 6.9. The change in urinary citrate excretion corresponding to these pH changes was an increase of 153 to 171 mg. The addition of orange juice increased the basal pH values by 0.3 to 0.6 units, to averages of 6.0 to 6.2, and the urinary citrate excretions by 63 to 152 mg.

Probably the suggestion that urinary excretion of citrates is related to the pH of the urine was first made by Östberg ('31). He observed that ingestion of either sodium citrate or sodium bicarbonate increased the citrate excretion, and concluded that the more alkaline urine was responsible for the increase in both cases. His experiments have been extended and his conclusions confirmed by others.

In Lanford's study ('42) using grapefruit juice as the source of citric acid, she found for the 10 subjects on controlled intake, an increase in the pH of the urine of 0.53 ± 0.11 units over the average of 6.00 on the control days. She gives statistical evidence that the increased citrate excretion and the rise in urinary pH were interdependent.

Schroeder and Smith ('43), in a study with rats, showed that on two isocaloric diets, containing 6 and 27 mg. of citric acid per period, the addition of graded amounts of sodium bicarbonate resulted in the excretion of similarly graded amounts of citric acid, independent of the citric acid intake. They have suggested that there is a direct relationship between the amount of alkali administered to the rat, and the excretion of endogenous citric acid, and that the total urinary citric acid is dependent on the degree of alkalosis.

In the present study the increase in the intake of citrates was, for each child, about 80 mg. more when orange juice was given than on the potassium citrate supplement, but the excretion was decreased by 44 to 95 mg. It has been pointed out that the pH of the urine was less alkaline with the orange juice than with the potassium citrate supplement. These observations confirm the conclusion that the acid-base relationship is more important than the citrate content of the diet in controlling citrate excretion.

SUMMARY

The citric acid metabolism of four preschool children was studied over an experimental period of 16 weeks. The children were on a controlled diet which was supplemented with 100 mg. of ascorbic acid, 3.38 gm. potassium citrate, or equivalent amounts of orange juice as indicated. The results were as follows:

1. On the basal diet the four children excreted an average of 55, 93, 100 and 247 mg. citric acid in the urine, or 3, 6, 7, and 13 mg./kg. of body weight.
2. The addition of 100 mg. of ascorbic acid to the basal diet did not cause consistent changes in citrate excretion, and no relationship was found between ascorbic acid "utilization" and citrate excretion.
3. The addition of 3.38 gm. of potassium citrate to the basal diet, with or without the addition of ascorbic acid, caused a definite increase in excretion of citrates, consistent in amount for all four children, averaging 153 to 167 mg., or 7.1 to 8.1% of the added citrate intake.

4. The addition of an amount of orange juice containing an amount of citrate approximately equivalent to that in the potassium citrate supplement caused an average increase in citrate excretion of 63 to 126 mg. or 2.8 to 7.0% of the added citrate intake.

5. The increase in citrate excretion on the addition of potassium citrate or orange juice to the diet appears to be related to the rise in alkalinity of the urine rather than to the citrate content of the diet.

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FURTHER STUDIES ON CYSTINE, METHIONINE AND CHOLINE IN CHICK DIETS

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The dietary requirements of the chick for the sulfur-bearing amino acids are met by levels of 1.0 to 1.1% of cystine plus methionine, if half of this total is supplied by methionine, and the diet contains adequate amounts of choline (Grau and Almquist, '43). Under certain conditions, methionine and betaine (glycine betaine) can replace a portion of the choline required in the diet (Almquist and Grau, '44), but replacement of methionine by dietary excesses of choline and cystine has not been thoroughly investigated.

Results of further studies on the interrelationships of the sulfur-containing amino acids may now be reported. The chicks used were not depleted of choline as before, but were fed a practical rearing mash from the time of hatching until they were segregated into groups of 5 and given the experimental diets; otherwise, the methods and diets were the same as those previously detailed (Grau and Almquist, '43).[1] A summary of the results is given in table 1.

At the low level of 1.7 millimols of added sulfur amino acids with choline present, methionine or cystine elevated the growth rate from a basal value of approximately 1% to a suboptimal plateau value of approximately 4% (fig. 1, Grau and Almquist, '43). The apparent equivalence of cystine and methionine in these cases was due to an extreme deficiency of cystine and a suboptimal level of methionine in the basal diet. The deficiency could be relieved by additional methionine, which is a precursor of cystine, or by cystine itself. When 2.5 millimols were added as cystine only (table 1, diets 15 and 44), the growth rate remained near the 4% value even with a high level of choline. When 3.3

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[1] To facilitate understanding the significance of the data now being presented, a summary of the basal diet follows: isolated soybean protein 23, glucose 52.8, cellulose (Cellu Flour) 5, calcium gluconate 8, mineral mixture 4.24, cottonseed oil (Wesson) 5, cod liver oil 1, and crystalline sources of vitamin K, thiamine, riboflavin, pyridoxine, nicotinic acid and calcium pantothenate. Biotin was provided by a concentrate. The soybean protein provided 20 gm. of protein per 100 gm. of diet; the methionine and cystine contents of the basal diet were 0.3% and 0.05%, respectively.

millimols of cystine were added (diets 45 and 46), gains were not appreciably changed from the 4% value at any level of added choline chloride up to 0.6%. When half of this cystine was replaced by methionine (diet 20) the gain per day was distinctly higher. At the 4.2 millimol level of added cystine (diet 47) the gain per day was not appreciably raised over 4%, and here, again, an excess of choline did not allow better growth.

TABLE 1

Effect of various added levels of cystine, methionine and choline on the rate of gain of chicks fed a basal diet deficient in all three compounds.

DIET NUMBER	SUPPLEMENTS ADDED TO THE BASAL DIET [1]			TOTAL MILLIMOLS SULFUR AMINO ACIDS ADDED PER 100 GM. DIET	NUMBER OF GROUPS	AVERAGE PER CENT GAIN PER DAY
	l(–)-Cystine	dl-Methionine	Choline chloride			
14 [2]	0.20	...	0.20	1.7	2	4.0
15 [2]	0.30	...	0.30	2.5	1	4.0
44	0.30	...	0.60	2.5	1	4.1
45	0.40	...	0.20	3.3	1	3.6
46	0.40	...	0.60	3.3	1	3.9
20 [2]	0.20	0.25	0.20	3.3	1	5.2
47	0.50	...	0.60	4.2	1	4.1
48	0.60	...	...	5.0	1	0.7
49	0.60	...	0.05	5.0	2	3.8
50	0.60	...	0.20	5.0	3	4.1
51	0.60	...	0.40	5.0	2	4.3
52	0.60	...	0.60	5.0	2	4.2
53	0.50	0.12	0.20	5.0	2	5.3
22 [2]	0.20	0.50	0.20	5.0	1	6.0
54	0.20	0.50	0.60	5.0	1	5.8

[1] Expressed in % added to diet.

[2] From Grau and Almquist ('43). The diet numbers were assigned previously. Chicks in these groups had been depleted of choline to a perotic stage.

When 5 millimols of cystine were added (diets 49, 50, 51 and 52), gains were essentially the same as before. The levels of choline chloride in these diets varied from 0.05 to 0.60%. The very poor growth with diet 48, to which no choline had been added, indicates that an extreme choline deficiency existed in the basal diet. When as little as 0.1% cystine was replaced by an equivalent amount of methionine, gain was increased to 5.3% (diet 53), while increasing the methionine still further (diets

22 and 54), resulted in gains of approximately 6%, the best rate obtained with this type of diet.

The data of table 1 show that gradual increases of cystine up to 3 times 1.7 millimols of added amino acids, and simultaneous increases of choline up to 12 times the minimum amounts which would support the plateau value of growth rate did not appreciably increase this growth rate above 4%.

These results, together with our former reports, present a fairly clear basis of understanding the interrelations of cystine, choline and methionine in the chick. During acute deficiencies of cystine and choline the chick effects the best adjustment possible in utilizing methionine for all three requirements. Whenever methionine must be partially utilized to fulfill the functions of cystine and choline, additions of cystine and choline may promote additional growth through sparing methionine. When this sparing action has been completely fulfilled at any suboptimal level of methionine, further additions of cystine or of choline are of no avail. Growth then cannot be accelerated except by additions of methionine. It is probable, however, that for each increment of methionine, small increases of cystine and of choline will show favorable effects on growth provided the amounts already present are balanced with the level of methionine.

Much has been implied concerning the possibility of using choline to improve diets in cases of inadequate methionine content. It is now evident that favorable effect from such supplementation could only be expected under circumstances where methionine is necessarily being used to provide some of the functions of choline in the diet, or in other words, where there is also a labile methyl deficiency. Our data show distinctly that an inadequate level of methionine cannot be compensated for by excessive levels of cystine and choline, and there is no evidence for a methionine synthesis (or interference with metabolic methionine destruction) through the presence of surpluses of these compounds in the diet.

SUMMARY

Through the use of a diet deficient in methionine and cystine, experiments were conducted in which the levels of cystine were increased gradually up to the optimal sulfur amino acid content, with various levels of choline additions. With levels of choline chloride as high as 0.6%, the growth obtained was no better than 4% per day, while substitution of part of the cystine by methionine so that the total methionine content of the diet was 0.55%, gave optimal gains of 6% per day.

Cystine apparently cannot compensate for a methionine deficiency, even if high levels of choline are employed.

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THE BASAL METABOLIC RATE OF THE AMERICAN NEGRO, WITH PARTICULAR REFERENCE TO THE EFFECT OF MENSTRUATION ON THE FEMALE

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THREE FIGURES

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A review of the literature on the determination of the effect of the menstrual cycle on basal metabolism of women yields conflicting evidence. The most general agreement seems to be on the existence of the premenstrual rise with a lowering during the actual menstruation and in the immediately postmenstrual period. This has been emphasized by Snell, Ford and Rowntree ('20), Rowe and Eakin ('21), and Wakeham ('23). Similar relationships have been reported by Kunde ('23), Collett and Liljestrand ('24), Hafkesbring and Collett ('24), Benedict and Finn ('28), Gustafson and Benedict ('28), Rogers and Flemming ('28), Hitchcock and Wardwell ('29), Udaondo ('32), Conklin and McClendon ('30), Wible ('31), and Arvay and Meyer ('32).

Other investigators have concluded that there is no demonstrable effect. Chief among these may be listed Salomon ('05), Zuntz ('06), Gephart and DuBois ('16), Wiltshire ('21), Blunt and Dye ('21), Lantz ('25), and Sandiford, Wheeler and Boothby ('31).

The work of Zuntz was done on two subjects only, while Gephart and DuBois reported three tests on a single subject. The validity of these results may be questioned from the paucity of data submitted. Wiltshire reported conflicting results on five subjects. The low values in metabolic rate during menstruation reported by Blunt and Dye were considered within the range of variation that may occur at any time. Lantz also reported negative results from a study of a healthy woman, while Sandiford, Wheeler and Boothby found no change in heat production, which can be considered indicative of a fundamental change in the intensity of the oxidation processes.

This study aimed at determining the periodic variation (if any) in the basal metabolic rate of Negro women during the menstrual cycle and at ascertaining whether their values differ enough from the standard value for white North Americans to suggest a racial factor in basal metabolism.

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EXPERIMENTAL PROCEDURE

One hundred and eighty-two basal determinations were made on twenty-seven healthy women between the ages of 17 and 35 years, inclusive. All tests were carried out on subjects in the post-absorptive state, after a night's rest in the metabolism laboratory. A Benedict-Roth-Collins metaboler with a kymograph attached was used in all cases. All calculations were made from the graphic record of the respiration of the subjects. In the computations, the DuBois height-weight formula was used for surface area determinations. For normal standards the Boothby and Sandiford modified form of the DuBois normal standards was used.

Tests were made before, during and after menstruation with an average interval of 7 days, except for two subjects for whom daily variations are reported. Thirty basal metabolism determinations were also made on fourteen Negro men between the ages of 18 to 24 years, inclusive.

RESULTS

Data for all subjects with respect to the average basal metabolic rate expressed as percentage above or below normal and the number of tests yielding this average are given in table 1.

TABLE 1

Basol metabolism of Negro women and men compared with the DuBois standards as modified by Boothby and Sandiford.

WOMEN						MEN		
Subject	No. of tests	Comparative average basal metabolic rate	Subject	No. of tests	Comparative average basal metabolic rate	Subject	No. of tests	Comparative average basal metabolic rate
1	6	– 11.9	16	23	– 13.34	1	2	– 9.2
2	5	– 16.64	17	6	– 7.2	2	3	– 16.3
3	9	– 14.73	18	9	– 14.72	3	2	– 3.2
4	5	– 13.96	19	7	– 15.0	4	2	– 9.7
5	5	– 11.88	20	4	– 13.32	5	2	– 11.8
6	5	– 19.56	21	2	– 7.66	6	2	– 3.6
7	3	– 8.43	22	3	– 4.31	7	2	– 8.7
8	5	– 4.65	23	3	– 17.13	8	2	– 15.3
9	5	– 13.32	24	5	– 22.06	9	2	– 11.8
10	8	– 18.02	25	4	– 9.96	10	2	– 11.8
11	3	– 20.1	26	6	– 6.56	11	2	– 16.0
12	5	– 15.48	27	4	– 14.43	12	2	– 20.8
13	8	– 19.65				13	2	– 11.2
14	26	– 18.62				14	2	– 22.3
15	8	– 21.6						

Figure 1 represents a composite smoothed frequency polyogon for all female subjects in which the average daily values were plotted against the basal metabolic rate before and after menstruation. Individual daily variations on two subjects are given in figures 2 and 3.

Pulse, temperature and blood pressure are not recorded here, as they were within normal limits.

FIGURE 1.
AVERAGE BASAL METABOLIC RATE OF 27 NEGRO WOMEN BEFORE, DURING AND AFTER MENSTRUATION

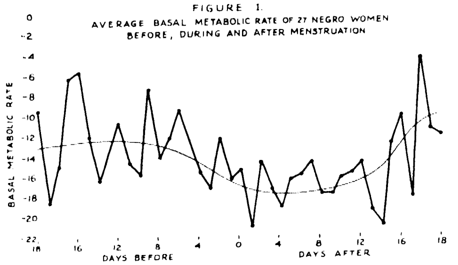


FIGURE 2
SUBJECT NO 14 DAILY VARIATIONS

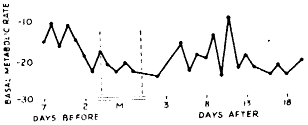
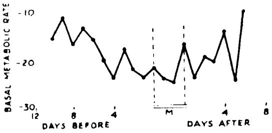


FIGURE 3
SUBJECT NO 16 DAILY VARIATIONS



DISCUSSION OF RESULTS

The results herewith presented indicate that Negro women give a lower basal metabolic rate than the value reported for white women of North America. The average deviation from the DuBois standards as modified by Boothby and Sandiford was — 14.6%.

Dr. Alberta Iliff, writing on behalf of Dr. Robert C. Lewis and his associates, of the University of Colorado School of Medicine, states (unpublished communication):

"We have made determinations of basal metabolism on men and women between the ages of 15 and 40 years. Although the number of tests is too few to make a statistical analysis, the mean values were definitely lower than the standards of Boothby, Berkson and Dunn

(Am. J. Physiol., vol. 116, p. 468, 1936). Consequently, the Department of Biochemistry has recently adopted standards of reference which are the Boothby, Berkson and Dunn standards less 10%. These revised standards agree well with the results which we have found.''

If these observations should prove to be generally valid, it would indicate that the basal metabolic rates of Negroes which we have determined are not significantly lower than those of white subjects.

In the selection of the male subjects, care was used in order to ensure a varied group, such as athletes in training, manual workers on the school farm, and students not engaged in any extra-curricular activity.

Considering the type of subjects, the co-operative spirit, and the number of tests made on each subject, it is felt that the data represent the true metabolic level as nearly as could be desired. The reported effects of the menstrual cycle on basal metabolism were confirmed. These consist essentially of a pre-menstrual rise in basal metabolism with a lowering during actual menstruation and in the immediately post-menstrual period. This is shown in the composite graph (fig. 1) for all subjects in which the average daily values were plotted against the basal metabolic rate before and after menstruation.

SUMMARY

1. The basal metabolic rates of twenty-seven women and fourteen men were determined by the Benedict-Roth-Collins metabolism apparatus and found to be 14.8 and 12.44%, respectively, below the Boothby and Sandiford modified form of the DuBois standards.
2. The reported effects of the menstrual cycle on basal metabolism (a pre-menstrual rise with a lowering during the actual menstruation and in the immediately post-menstrual period) were confirmed.

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STUDIES OF CALCIUM AND PHOSPHORUS METABOLISM IN THE CHICK

III. SOME TIME RELATIONSHIPS IN THE ACTION OF VITAMIN D[1]

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(Received for publication October 13, 1944)

The mode of action of a number of the vitamins is now comparatively well understood. Nevertheless, the mode of action of vitamin D remains obscure. The literature on this subject has been reviewed by Reed, Struck, and Steck ('39) with these conclusions: (1) The primary function of vitamin D is to render calcium and phosphorus available to the tissues, (2) it also makes the tissues more receptive to these ions, and (3) the manner in which this is accomplished remains to be elucidated.

More recently Nicolaysen and Jansen ('39) demonstrated that there was no difference in the density of calcification in albino rats whether they received vitamin D or not, provided that adequate amounts of calcium and phosphorus were actually brought to the blood. There was, however, some histological difference in the structure of the bones of the animals which received vitamin D. Cohn and Greenberg ('39), using P^{32} as a trace element, concluded that vitamin D acts by aiding the conversion of organic to inorganic phosphorus. Morgareidge and Manly ('39), also using P^{32} as a trace element, concluded that the action of vitamin D is not limited to the control of the intestinal absorption of calcium and phosphorus.

Since the manner in which vitamin D acts still remains understood only in a very general way, it was felt by the authors that it might be worthwhile to make studies of mineral retentions following single oral dosages of vitamins D_2 and D_3 to the chick, with particular reference to these considerations: (1) How soon after the vitamin dosages an improvement in mineral balances becomes evident, (2) how long the favorable effect of the dosages persists, and (3) whether there is any quantitative relationship between the level of mineral retention and the amount of vitamin D available to the chicks at different times. It was also hoped that the information so obtained might reveal significant differences in the action of vitamins D_2 and D_3 which would aid in explaining the fact that the former is only about 2 to 3% as effective as the latter in the chick. Previous papers of this series (McChesney, '43 b, '43 c) have shown that this phenomenon may be accounted for in part by the better absorption of vitamin D_3, but even after allowance for this is made, the vitamin D_3 remains about twenty times as effective as D_2.

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[1] Presented before the Division of Biological Chemistry of the American Chemical Society at Cleveland, April 4, 1944.

Studies of the type outlined above have not been possible thus far because of the lack of definite information about (and methods for the study of) the metabolism of vitamin D in various species. Knudsen, Remp, and Barlow ('41) studied the metabolism of several forms of vitamin D in the albino rat and found that after 10 consecutive days of oral dosage, only a fractional part of 1 day's dosage could be recovered from the carcasses on the eleventh day, indicating very rapid excretion and/or inactivation. McChesney[2] has studied the metabolism of massive (hypercalcemic) doses of vitamins D_2 and D_3 in the chick. The former was found, under certain conditions, to disappear from the organism at a rate considerably exceeding that of the latter.

OUTLINE OF EXPERIMENTAL PROCEDURES

Male white Leghorn chicks were received in the laboratory on the second day of life. They were immediately offered water and a vitamin D-free chick ration, which is slightly modified from that of Hart, Kline, and Keenan ('31); i.e., 2 parts of corn oil are substituted for 2 parts of yellow corn. On the sixth day of life the chicks were divided into three groups of twenty, and one group of forty. The purpose was to use the first three groups for mineral balance studies, and the last for studies of vitamin D_2 metabolism. Accordingly, for the first three groups, the food consumption was now determined, the excreta were collected, and distilled water was substituted for tap water. At first the intervals studied were 3 or 4 days in duration, but later in the experiment, when it was desirable to study short-time variations, periods as short as 1 day were used. The chicks were weighed at the beginning and end of each period for which the mineral balances were to be determined. Food and excreta were analyzed for calcium and phosphorus by methods which have been outlined previously (McChesney, '43 b); the diet was found to contain 0.86% Ca, 0.68% P. From the data so obtained the calcium and phosphorus balances were calculated in terms of milligrams retained per 100-gm. chick per day. The weight factor was introduced in order to eliminate the effect of growth as the experiment proceeded.

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[2] To appear in Proc. Soc. Exp. Biol. and Med.

On the twentieth and thirty-fourth days of life the chicks were given oral supplements (by stomach tube) as follows: Group A, propylene glycol, 0.2 ml.; group B, vitamin D_3, 60 I.U. in 0.2 ml. propylene glycol; group C, vitamin D_2, 2400 I.U. in 0.2 ml. propylene glycol; group D (40 chicks) same as group C.

Metabolism of vitamin D_2. At selected intervals after the vitamin supplementations (table 1) groups of four chicks were taken from group D for vitamin D assay. Since these chicks were on the same regimen as group C, it was considered that their average body content of vitamin D would be the same as that existing in group C at the same time. The four chicks were at once digested in boiling alcoholic KOH, and extracts

TABLE 1

Average body weights and mineral retentions of chicks receiving oral supplements of vitamin D at selected intervals.

OBSERVATION PERIOD, DAYS OF LIFE	GROUP A			GROUP B			GROUP C			BODY CONTENT OF VITAMIN D_2[3, 4]
		Retention[2] of			Retention of			Retention of		
	Av. wt.[1]	Ca	P	Av. wt.	Ca	P	Av. wt.	Ca	P	
7–10	71	68.6	46.3	71	90.0	51.4	71	81.5	46.2	
11–14	90	37.2	28.2	89	46.4	36.6	90	56.4	39.8	
15–17	102	26.7	20.0	103	24.0	22.0	106	30.6	28.3	
18–20	110	24.6	18.9	115	29.6	29.0	118	22.3	22.3	
21[5]	117	29.0	17.1	122	39.5	28.0	126	35.4	24.5	625
22	119	30.3	17.8	126	73.4	45.6	131	63.3	41.2	787
23	119	23.6	16.0	131	55.8	32.8	136	52.8	31.8	...
24–25	123	15.2	15.2	138	54.5	39.6	144	46.1	35.1	460(25)
26–27	130	20.8	18.6	150	47.1	35.3	158	34.3	25.9	365(27)
28–29	135	19.2	16.0	159	34.7	30.8	170	26.3	23.8	...
30–31	138	17.5	14.1	166	25.0	18.8	180	24.9	20.6	262(30)
32–34	145	23.8	19.4	179	19.4	18.1	192	21.8	18.3	...
35[5]	151	25.0	21.4	188	37.9	27.2	201	44.4	34.1	1050
36	153	21.0	17.4	191	44.5	32.1	208	36.4	24.2	...
37	156	15.6	14.4	197	52.4	35.2	212	34.8	24.5	567
38–39	160	12.0	10.3	212	43.4	31.6	221	31.1	23.3	435(39)
40–41	164	12.4	11.8	224	24.5	20.5	235	27.6	23.8	470(41)
42–43	168	8.6	7.3	231	19.3	15.7	241	23.1	16.7	...
44–45	169	7.6	6.5	246	21.2	17.5	254	18.3	14.7	325(45)

[1] Grams.

[2] Calculated as milligrams of the element retained per 100-gm. chick per day.

[3] Of group D, which received the same treatment as group C.

[4] The figures in parentheses indicate the day of life on which the observations were made where such would otherwise be ambiguous.

[5] Vitamin supplements given at the beginning of this 24-hour period as follows: Group A, none; group B, 60 I.U. of vitamin D_3; group C, 2400 I.U. of vitamin D_2.

were prepared according to methods already described (McChesney, '43 a). The ether extract so obtained was evaporated and taken up in a volume of oil such that the concentration of vitamin D was estimated to be 16.7 I.U. per milliliter. (These volumes varied from 120 ml. 24 hours after supplementation down to 15 ml. on the tenth day after supplementation.) Then an equal volume of an oily solution of vitamin D_2 known to contain 16.7 I.U. per milliliter was added. This was necessary in order to guarantee sufficient material to complete the bioassays in case the first test fell outside the readable range. This procedure, however, has the effect of doubling the experimental error when the final estimate of the vitamin content of the chicks is made.

In the case of the 7- and 10-day interval extracts, it was impossible to reduce the ether solutions to the desired volumes by evaporation, and the preparations so obtained were not homogeneous. The volume of known vitamin D solution added was the same as originally planned, but the final volume of the mixture instead of being 30 ml., for example, was as much as 95 ml. Thus, in the bioassay it was necessary to administer as much as 0.3 ml. per rat per day (by stomach tube) instead of the usual 0.1 ml. The material appeared to be somewhat irritant, and the animals did not eat well. This resulted in some "starvation cures", and the results for the 7- and 10-day intervals after supplementation may be somewhat high.

Unfortunately the prophylactic dose of vitamin D_3 is entirely too small to permit a parallel study of its metabolism. Even 24 hours after administration sufficient material could not be extracted to permit a bioassay.

RESULTS AND DISCUSSION

The numerical data are given in table 1.

Even before any vitamin supplements were given, some variability in the mineral balances of the different groups was evident. However, the observations for days 15 through 20 were about as uniform as could be expected, and the trend indicated that a "resistance level" had been reached. This is confirmed by the fact that group A, which received no vitamin D at any time, maintained essentially this level of mineral retention through the thirty-sixth day of life. The most reasonable interpretation of the behavior of the birds through the preliminary control period (days 6–20) is that they inherited a considerable supply of vitamin D, and that this permitted them to maintain satisfactory mineral balances through the first 14 days of life. By this time they had apparently exhausted all but a small part of the inherited D and were unable to maintain mineral balances above the resistance level. Only after a long time (group A, days 37–45) was the resistance level permanently broken; the birds then went into terminal rickets and deaths began to occur. A reasonable interpretation of this would be that during the 14- to 36-day interval they still had available a very small amount of vitamin D which they conserved tenaciously, but which was finally exhausted.

The first supplementation with vitamin D_2 or D_3 produced only a slight improvement in mineral balances within the first 24 hours. However, this cannot be interpreted to mean that there is a latent period before vitamin D becomes effective since part of the excreta collected for this period represents food ingested during the preceding 24 hours; furthermore, the second vitamin supplementation did produce a more evident immediate improvement. The positive effect from the first supplementation lasted 8 days, when the resistance level had again been reached. The duration of the positive effect from the second supplementation was barely 7 days, provided the same resistance level is taken as a reference point rather than the value observed in the control chicks at that particular time.

The effect of the second supplementation was quantitatively inferior to the first in terms of mineral balances. This seems logical in view of the fact that the supplements were the same in both cases, but in the meantime the chicks had increased in weight by about 80%.

In view of the previously mentioned differences in rates of inactivation of vitamins D_2 and D_3 in the chick, it was thought that some characteristic difference in the mineral retention curves for the two products might be found. (For example, following administration of vitamin D_2 the same total retention might be accomplished in a shorter period of time.) However, there is nothing in the character of the data to indicate that vitamin D_2 and D_3 affect mineral retention differently either qualitatively or quantitatively, barring the fact that forty times as much of the former is required to produce the same quantitative effect. With both preparations positive retentions of about the same magnitude were observed for the same time interval after supplementation. The results, therefore, offer no clue as to the cause of the remarkable difference in activities of the two preparations in the chick.

From the amounts of vitamin D_2 found in the chicks 24 hours after the administration of 2400 I.U., the amount which was actually absorbed may be calculated roughly. The figure for day 21 is clearly too low, as a value of around 900 I.U. would fit in better with the rest of the data. Extrapolating back to day 20, it would appear that a value of about

1000 I.U. should have been found immediately following absorption. Of this amount about 250–300 I.U. may be assumed to have been already available to the chicks since they were then at what has been termed the "resistance level" of mineral retention (as they were on day 30 when the body content of vitamin D was 262 I.U.). Therefore, roughly 700–750 I.U. or 30% of the dose administered was absorbed. The body content of 1050 I.U. on day 35 indicates the same general conclusion, with allowance for a carry-over of about 250 I.U. from the first supplementation. However, it must be kept in mind that this seemingly large amount of 250 I.U. represents only 5 I.U. in terms of natural vitamin D, or less than 2 days' requirement for a chick.

The vitamin D was inactivated at a rate of 10 to 15% per day during the time that its metabolism was studied. This rate of inactivation is materially less than has previously been found for massive doses of the vitamin.[3]

The mineral balances, taking the expected daily variations into account, are in general directly related to the amount of vitamin D available at the time. The most interesting phenomenon revealed by the work is the minimum level of calcium and phosphorus retention (about 20 mg. per 100-gm. chick per day) which the birds were able to maintain for a considerable period of time without receiving any vitamin D. This could, of course, be due to an extremely small amount of vitamin D in the diet, an amount which finally became completely inadequate when the birds became quite large.

SUMMARY

The baby chicks used in this study had a supply of vitamin D adequate for about 14 days of life. Their calcium and phosphorus retentions then reached a level of about 20 mg. per 100-gm. chick per day, a level which they were able to maintain for 3 weeks. After this there was a decline to very low levels as the birds went into a state of terminal rickets. However, chicks which received oral supplements of vitamin D_2 or D_3 showed an immediate increase in calcium and phosphorus retentions. The improvement was evident for 7 or 8 days, after which the chicks returned to the same low level of retention. A second vitamin supplementation of the same magnitude produced a positive, but quantitatively inferior, effect, probably due to the intervening growth of the birds. Following supplementation, the increased level of mineral retention was directly related to the amount of vitamin D available. The body content of vitamin D_2 following an oral dose of 2400 I.U. increased to about

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[3] See footnote 2, p. 230.

1000 I.U., and, in the ensuing 10 days, fell to about 250 I.U. This residue, equivalent to about 6 I.U. of vitamin D_3, appeared to be rather tenaciously conserved.

ACKNOWLEDGMENT

The technical assistance of Donald Seppelin and Mrs. Charles Miller is hereby gratefully acknowledged.

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STUDIES ON THE COMPARATIVE NUTRITIVE VALUE OF FATS

V. THE GROWTH RATE AND EFFICIENCY OF CONVERSION OF VARIOUS DIETS TO TISSUE IN RATS WEANED AT 14 DAYS[1]

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TWO FIGURES

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There have been several reports in the literature from the Wisconsin group that butterfat possesses certain saturated long-chain fatty acids, not present in vegetable oils, which accentuate the growth of young rats during the first several weeks following weaning although the variation in growth response may disappear after 6 weeks (Schantz, Elvehjem and Hart, '40; Schantz, Boutwell, Elvehjem and Hart, '40). In a later study (Boutwell et al., '43a), it was reported that this particular superiority in growth-promoting property of butterfat over corn oil is only to be noted in those diets where the sole carbohydrate is lactose. This superiority of butter disappeared when lactose was replaced by glucose, sucrose, dextrin or starch, and actually slightly greater gains (though not statistically significant ones) were noted under these circumstances during the 6-week interval following weaning. In an extension of this work (Boutwell et al., '43b) with synthetic diets where the lactose or other carbohydrates made up 48% of the diet instead of 32% used in the earlier tests, no differences were noted between butter or lard and a series of vegetable fats when a mixture of carbohydrates was employed; however, in most cases, poorer growth was obtained when lactose alone was used. With seven types of oleomargarines of animal and vegetable origin, the growth during a 6-week period averaged 6.4% lower than butter when the high lactose was employed and was identical with it when the mixed carbohydrate diet was fed.

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[1] This work was carried out under a research grant from The Best Foods, Inc. The authors wish to acknowledge the helpful advice of Prof. Anton J. Carlson of the University of Chicago, of Prof. Arthur W. Thomas of Columbia University, and of Dr. H. W. Vahlteich of The Best Foods, Inc., during the course of the experiments.

In a previous report from this laboratory (Deuel et al., '44), no differences were noted in the growth of 21-day weanling rats fed a diet where lactose was the sole carbohydrate at the level found in milk, irrespective of whether the fat added to the mineralized skimmed milk powder was a butter, a margarine, or corn, cottonseed, olive, peanut or soybean oil, all of which were fortified with vitamins A, D and E. The ratios of food consumed to increase in body weight were similar when the different diets were employed, which would indicate a similar efficiency in utilization. It was also found (Deuel and Movitt, '44) that rats prefer a butter diet or one flavored with diacetyl or commercial butter flavor to one where this flavor is not present. It was suggested that superior growth response of animals fed on a butter diet over those on other fats which are unflavored in experiments where ad libitum feeding is employed may well be ascribed to a greater food consumption.

Another condition where the superiority of butterfat over the vegetable oils has been reported to be enhanced, is in rats prematurely weaned (Boutwell et al., '43a). The Wisconsin investigators found that the variations in growth response had practically disappeared in rats which were not started on the diet until they were 30 days of age; on the other hand, they were accentuated in rats weaned at 14 days over those weaned at the usual 21-day period. These results are interpreted as indicating that the rat gradually stores the growth-promoting compounds (saturated long-chain fatty acids) during nursing as well as when fed the stock ration so that an adequate reserve is available when these substances later are withheld from the diet. It is suggested that it is difficult to deplete rats of this factor when they have had access to the stock ration for 10 days. Zialcita and Mitchell ('44), however, have failed to confirm the superiority of butterfat over corn oil on the subsequent growth of rats weaned as early as 7 days and fed in both cases until 21 days of age, an artificial liquid milk diet containing butterfat or corn oil followed by a synthetic solid milk diet with these fats thereafter. The present tests also are designed to give further information on the relation of age to nutritive value of the fat with rats weaned at 14 days of age.

EXPERIMENTAL

The tests were carried out essentially the same as in the earlier studies (Deuel, Movitt, Hallman and Mattson, '44), using the same strain of rats. In order that the rats be sufficiently developed to make possible a survival after weaning at 14 days, the growth was stimulated by reducing the litters to two rats at 3 days of age. When the litters were 7 days old, the mothers which had previously received our stock diet were placed on the butter, margarine, corn oil, cottonseed oil, peanut oil or soybean oil diet described below. When the young were weaned, one was placed on one of the six diets listed above and the litter mate was assigned to the butter diet as a control. In the case of the group where the mothers had been placed on the butter diet, the second rat in the litter was placed on the cottonseed oil diet. During the first week cotton batting was placed in their cages. The temperature of the room was maintained throughout at about 72°C. Small Petri dishes were employed for feeding during the first week. Because of the spilling of the food during the first week, it was not possible to obtain accurate values of the amount eaten until the second week. Practically all of the rats survived and gained on an average of 5 to 8 gm., which is probably less than they would have had they remained with their mothers.

Experiments were completed on 120 male rats and 112 female rats from our stock colony. In any tests where diarrhea developed, both litter mates were discarded. The diet differed from that used for series II but was identical with that used for series III (diet 53 previously listed as diet II) in which whole butter or whole margarine was used instead of the separated fat, and additional water was added to the oil diets. The following proportions of mineralized skimmed milk powder and vitamin-fortified fats were used: Diet 54, skimmed milk powder 64.5 parts, oil or fat 26.9 parts, water 8.6 parts (caloric value 4817 Cal. per kilo.); diet 53, skimmed milk powder 70.6 parts, whole butter or whole margarine 29.4 parts (caloric value 4690 Cal. per kilo.). Care was taken to use only fresh fats. Diets were prepared weekly, and stored in a refrigerator; they were fed ad libitum. The diets were flavored with commercial butter flavor.[2] The present tests are designated as series IV.

RESULTS

The averaged weights of the rats receiving the different diets are illustrated in figure 1 for the males and in figure 2 for the females. The values are shown for the start (14 days) for the 1st, 3rd, 6th and 12th week thereafter. The weights of the rats (listed as series III) which received similar diets but which were weaned at 21 days (Deuel, Movitt, Hallman and Mattson, '44) are included for comparison at corresponding ages.

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[2] B. F. A. supplied by Verlay Products Corporation, 1621 Carroll Ave., Chicago, Ill., was used in a ratio of 4 parts per 1,000,000 parts of fat.

TABLE 1

The average weight increases, food consumption, caloric intake and efficiencies in male and female rats weaned at 14 days.

DIET	AVERAGE OVER 2ND TO 12TH WEEK INCLUSIVE						AVERAGE EFFICIENCY ((GM. GAIN/CAL. CONSUMED) × 100)) OVER FOLLOWING WEEKS INCLUSIVE							
	Weight increase[1]		Total food		Total calories		Male rats				Female rats			
	M	F	M	F	M	F	2–3	4–6	7–12	2–12	2–3	4–6	7–12	2–12
	gm.	*gm.*	*gm.*	*gm.*										
Butter	235.9 ± 14.9	145.6 ± 3.8	934	721	4385	3380	9.8	5.3	4.48	5.38	10.8	5.6	2.35	4.32
Cottonseed oil	244.1 ± 5.1	143.3 ± 3.8	818	690	3940	3325	10.5	7.5	4.80	6.19	9.5	5.1	2.74	4.32
Corn oil	234.4 ± 5.1	143.0 ± 3.3	808	665	3890	3205	9.8	7.8	4.44	6.03	10.4	5.8	2.43	4.45
Butter	226.9 ± 8.8	143.2 ± 4.0	879	703	4120	3300	9.3	6.9	4.15	5.52	9.9	5.5	2.28	4.34
Cottonseed oil	247.5 ± 10.1	141.0 ± 5.3	838	694	4030	3340	10.0	8.1	4.55	6.14	9.0	5.1	2.74	4.22
Butter	256.3 ± 10.0	148.0 ± 4.7	922	733	4320	3300	10.0	7.2	4.43	5.93	9.6	5.5	2.35	4.48
Margarine	230.8 ± 4.4	137.2 ± 5.1	884	708	4150	3320	9.2	7.2	4.22	5.57	10.0	5.0	2.28	4.13
Butter	234.6 ± 5.8	137.6 ± 5.3	885	735	4155	3445	9.7	6.6	4.57	5.64	9.2	5.2	2.18	3.99
Peanut oil	234.7 ± 10.6	138.1 ± 2.8[2]	831	704[2]	4000	3390[2]	10.5	6.8	4.48	5.87	8.9	4.7	2.61[2]	4.08[2]
Butter	237.6 ± 6.8	157.9 ± 7.0[2]	889	747[2]	4170	3505[2]	9.7	6.3	4.48	5.69	9.8	5.6	2.61[2]	4.51[2]
Soybean oil	244.8 ± 5.4	156.6 ± 6.3[3]	812	678	3910	3265	11.0	8.0	4.40	6.23	9.5	6.2	2.92	4.79
Butter	233.7 ± 6.8	145.4 ± 5.8[3]	854	688	4010	3220	9.8	6.3	4.36	5.83	10.5	5.4	2.62	4.52
Average butter tests	237.5	145.8			4190	3360	9.7	6.5	4.42	5.67	10.0	5.5	2.38	4.35
Average non-butter tests	239.4	143.0			3990	3310	10.2	7.6	4.72	6.50	9.6	5.3	2.50	4.33

The first diet listed in each group of two was the original diet of the mother before the rats were weaned.

[1] **Including the standard error of the mean calculated as follows:** $\sqrt{\frac{\Sigma d^2}{n}} / \sqrt{n}$ **where "d" is the deviation from the mean and "n" is the number of experiments.**

[2] **Eight experiments only.**

[3] **Eight experiments only with females throughout.**

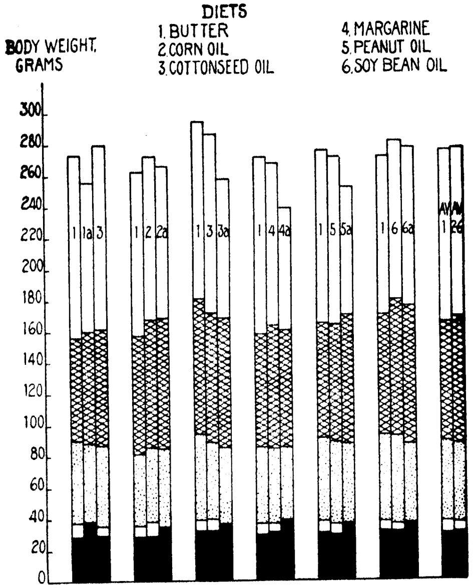

Figure 1

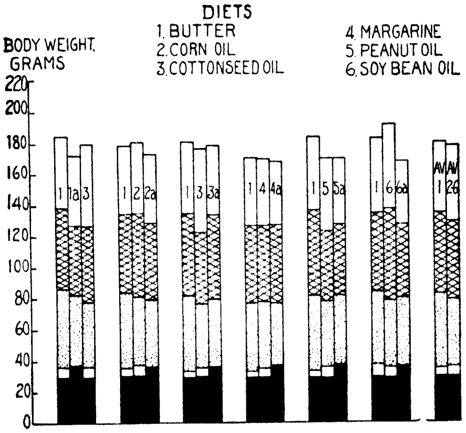

Figure 2

Fig. 1 The average body weight of male rats at weaning (14 days of age for series IV, 21 days of age for series III, designated by letter "a") in solid block, at 21 days (series IV only) to top of lower blank space, at 35 days (stippled), at 56 days (cross-hatched) and at 98 days (to top of upper blank space). Averages are for ten rats in all cases with series IV and for twelve to fourteen rats in various groups in series III. The average butter and average non-butter series (at extreme right) are for sixty rats each.

Fig. 2 The average body weight of female rats at weaning (14 days of age for series IV, 21 days of age for series III, designated by letter "a") in solid block, at 21 days (series IV only) to top of lower blank space, at 35 days (stippled), at 56 days (cross-hatched) and at 98 days (to top of upper blank space). Averages are for ten rats in all cases with series IV except experiments on peanut and soybean oils (eight rats each), and thirteen to sixteen rats in various groups in series III. The average butter and average non-butter series (at extreme right) are for fifty-six rats each.

The total gain in weight, food consumption and the caloric equivalent of the food are summarized for the 11-week period from 21 to 98 days of age. Because of the unavoidable spilling of food during the first week, the values for this period are not included. The efficiency of conversion of ingested food to body tissue is compared on the basis of the gain in weight per calories consumed. These data are summarized in table 1.

DISCUSSION

No differences were noted over a 12-week period in the growth rate of rats weaned at 14 days of age when placed on a diet containing mineralized skimmed milk powder and fat, irrespective of whether the latter was a butter, a margarine or such vegetable oils as corn, cottonseed, peanut or soybean, all of which had been fortified with vitamins A, D and E. Thus, the average total increase in weight of the male rats receiving the various diets was 244.7 gm. for the butter controls (sixty animals), and 245.5 gm. for the same number of litter mates which received one of the five other fats in place of butter. The corresponding increments in growth of the female rats were 151.7 and 149.0 gm., respectively, for fifty-six rats receiving the butter diet and the same number which ate the other fat diets. Although there are some variations in the average total gain in weight of the animals receiving the various fats and their butter diet controls, the differences are in no case significant statistically. In some cases the differences favor the vegetable oil group, while in some instances the opposite is true. As shown in figures 1 and 2, no appreciable variations were observed in growth rate between the groups receiving the various fats and butter at 1, 3 or 6 weeks. According to the results of Schantz, Elvehjem and Hart ('40), it is at these earlier periods that such differences should be noted.

The efficiency with which the food is utilized also shows no clear-cut differences between the butter diets and those containing the other fats. The over-all average for the males covering 11 weeks is 0.0567 and 0.0650 gm. gain per Calorie of butter diet and non-butter diet, respectively, that was consumed; for the females the corresponding values are 0.0435 and 0.0433 gm., respectively. The ratios between increase in body weight and caloric intake show approximately a similar relationship for the 2-3, 4-6 and 7-12 week periods.

As we have noted earlier (Deuel, Movitt, Hallman and Mattson, '44), the efficiency of conversion of ingested food to body tissue is less in females than in males and in both sexes becomes lower as the animals become larger. This is, of course, explained by the fact that the efficiencies are calculated on the gross energy consumption rather than on the net value after subtraction of that used for basal metabolism, specific dynamic action, etc. As the proportion of the total used for growth becomes smaller, the efficiency based on the gross intake will also become smaller. Furthermore, in the later periods the rate of growth becomes less, and the efficiency approaches a value of zero when growth ceases. This disproportion between caloric needs for basal requirements and growth accounts for the lower efficiency in the females where the growth rate is lower than the males in the later periods.

Although the reason for the difference in efficiency in our tests and those of Boutwell et al. ('43b) is not entirely clear, it may possibly be because of the lower proportion of lactose in our diet. Whereas these investigators report that the efficiency of corn oil was 31% less than butter during the first 3 weeks, in our tests it averaged 105% of the efficiency of the butter controls.

A lower absorption with a decreased efficiency would result if a diarrhea is present. In the few cases where diarrhea occurred in our tests, the experiments on both litter mates have been excluded from consideration, as they were in all probability from the results of the Wisconsin investigators. When the lactose content of the diet was increased to as high as 73%, we have found not only that a diarrhea may occur but also in many instances it may terminate fatally (Ershoff and Deuel, '44). Another possible explanation may be a strain variability in the animals used. Whereas weanling rats of the Long-Evans strain died on an average of 4.9 and 11.5 days after receiving diets containing 73.2% of beta-lactose or lactose (Ershoff and Deuel, '44), the average survival time for the University of Southern California strain was 22 days for beta-lactose and 26 days for the lactose group. Also, a similar variation in the period of incidence of a characteristic alopecia was noted between these two strains. In view of this one might also expect a variation with the Sprague-Dawley rats, the strain used by the Wisconsin investigators.

No marked variations in the average body weights are to be noted between the prematurely weaned rats and those of series III which were reported earlier. This would indicate that no harmful effects resulted from the shortening of the lactation period.

According to these results, aside from differences in vitamin content, corn, cottonseed, peanut and soybean oils and a margarine and a butter have essentially equal growth-promoting values when fed with lactose as the exclusive carbohydrate in the proportion found in milk. In confirmation of the work of Zialcita and Mitchell ('44) such a relationship also occurs in prematurely weaned rats.

SUMMARY

In experiments on 120 male and 112 female rats, the rate of growth of animals weaned at 14 days was found to be identical over a period of 12 weeks on diets of mineralized skimmed milk powder to which was added vitamin-fortified corn, cottonseed, peanut or soybean oil or a margarine as on similar diets containing butter as the fat. The efficiency of transformation of these diets to body tissue was also similar within experimental error.

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STUDIES ON CAROTENOID METABOLISM

V. THE EFFECT OF A HIGH VITAMIN A INTAKE ON THE COMPOSITION OF HUMAN MILK [1]

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The amount of vitamin A present in human milk is regulated by the diet in much the same way as in the case of cow's milk. Thus, on a vitamin A-low diet the milk may contain an insufficient amount of vitamin A to prevent avitaminosis. In fact, Thatcher and Sure ('32) have reported the death of an infant, who had been fed exclusively on breast milk from a malnourished mother, and in whom frank symptoms of vitamin A deficiency were apparent before death as well as at autopsy. On the other hand, milk from women on an average American dietary has been shown by Macy et al. ('27) to contain amounts of vitamin A sufficient to permit normal growth and reproduction in rats on an A-free diet supplemented with 2.5 or 3.0 ml. of pooled human milk daily. Dann ('36) found that although the vitamin A content was of the same order of magnitude in human and cow's colostrum, the vitamin A in human milk far exceeded that in cow's milk, the average vitamin A activity in 104 samples calculated for both carotene and vitamin A being 346 I.U. per 100 ml.

Although it is evident that the presence of adequate amounts of vitamin A in the diet will cause the transfer of vitamin A to the milk, there is a wide range of intake over which no parallel increase in vitamin A excretion is to be noted in the human as well as the cow. For example, McCosh, Macy, Hunscher, Erickson and Donelson ('34) found no rise in the vitamin A in the milk of three women who took 15 gm. of cod-liver oil daily in addition to an abundant and well-chosen diet. Moreover, there was no evidence of a deleterious effect on the fat content of milk as had previously been found to be the case for cows (Goldring et al.,

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[1] This investigation was made possible through grants from the Nutrition Foundation, Inc. We also wish to thank the Vitamin Oil Producers Institute for furnishing the vitamin A capsules.

'26). Dann ('36) also found no increase in the vitamin A content of colostrum or of mature milk in women whose diets were supplemented with cod-liver oil starting during pregnancy and continuing during the lactation period, but the dosages of cod-liver oil are not indicated. The failure to obtain an appreciable augmentation in the vitamin A in milk under such dietary regimes has been interpreted to mean that there is some factor which limits the amount of vitamin A which may pass into milk (Dann, '32).

However, the administration of what may be considered massive doses of vitamin A in the form of shark-liver oil to Guernsey and Holstein cows already receiving an excellent diet has been shown to result in a marked increase in the vitamin A content of milk (Deuel et al., '41, '42). In some cases the vitamin A of the butter fat was found to exceed 300 I.U. per gram in contrast to control values of about 40 I.U. per gram. Moreover, no decrease either in milk production or in the fat content of the milk was observed under these circumstances. In later work from this laboratory, similar effects were found on the vitamin A content of the hen's egg as a result of the administration of large amounts of this vitamin to hens (Deuel et al., '43). In both species of animals the quantity of vitamin A so transferred was roughly proportional to the dosage of vitamin A after the threshold had been exceeded; it was not until vitamin A in excess of 500,000 I.U. daily had been administered to cows and in excess of 3300 I.U. per day was fed to chickens that any increased vitamin A was to be found in the milk or eggs.

The present studies were designed to determine whether the human subject would react in a similar manner to dosages of vitamin A which are considerably higher than those usually regarded as adequate. By analogy from the cow experiments it was believed that an intake of 100,000 I.U. daily would be the level which should cause an increase in excretion of vitamin A in the milk of lactating women. Although there are some instances where toxic effects have been reported in animals when large dosages of vitamin A have been given over long periods, the results of Straumfjord ('42) where daily dosages of 100,000 I.U. and higher were given to patients in some cases continually over a period of 4 years without deleterious effects, would indicate that these levels could be used safely in women. Straumfjord ('40) also has given similar doses during 6 months or more of pregnancy where a beneficial effect on vernix caseosa was noted. These clinical reports as well as the earlier experiments on cows and chickens where considerably higher levels of vitamin A were administered over a number of months were taken as evidence that the dosages employed here were in the therapeutic range.

EXPERIMENTAL

The experiments were carried out on forty-two women who received vitamin A in amounts of 50,000, 100,000 or 200,000 I.U. each.[2] In addition, experiments were made on nine women who received vitamin A in a dosage of 200,000 I.U. daily for only 7 to 10 days prior to obtaining the sample. A total of 127 samples were obtained from these subjects. In most cases the vitamin A supplementation was started at 6 months of pregnancy and continued until lactation was voluntarily terminated.[3] The patients selected were Caucasian from the middle or upper economic bracket who had the desire to nurse their offspring and who had adequate breast tissue to make this possible. All of these patients were directly under the care of one of us (B.J.H.) during the time that this work was being carried on.

In addition, eighty-five samples of milk were obtained from normal patients in various stages of lactation who were not receiving any vitamin A other than that obtained in an adequate diet. These are listed as control experiments. All samples were obtained by breast pump. The samples were immediately brought to the laboratory, refrigerated and the vitamin A determination made on the following day. Vitamin A and carotene were in most cases determined in duplicate on the non-saponifiable residue. This was carried out as follows: after mixing 10 ml. of milk, 10 ml. of 95% ethanol and 2 ml. of 40% KOH, the mixture was saponified by refluxing for 30 minutes. It was then transferred to a continuous all-glass extraction apparatus with sufficient 50% alcohol to make a total volume of 80 ml. This was extracted with low-boiling Skelly Solve for 3 hours. All operations were carried out in a dark room.[4] After washing the petroleum ether three times with a total of 50 ml. of 1% HCl, it was dried overnight with anhydrous Na_2SO_4, filtered, evaporated to small volume on a water bath and the last traces of petroleum ether removed in the vacuum oven.[5] The residue was dissolved in 3 ml. of dried redistilled $CHCl_3$ and vitamin A determined by the Carr-Price method on a Klett-Summerson photoelectric colorimeter using a special 600-mμ. filter. Readings were made 1 minute after mixing with the $SbCl_3$ reagent, and vitamin A calculated from a standard curve which had been made using similar procedures on standard reference cod-liver oil. Correction was made for the effect of carotene. Carotene was determined on the combined $CHCl_3$ extracts in the same colorimeter using the blue filter (420 mμ.).

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[2] Prepared by Gelatin Products, Inc., Detroit, for the Vitamin Oil Producers Institute.

[3] The subjects were instructed to take the capsules daily but we cannot be certain that in all cases they were conscientious in taking their supplements regularly. Deviations in this may account for some of the variations obtained.

[4] The light bulbs were covered with a special lacquer which prevented any destruction of vitamin A. It was kindly furnished us by Mr. A. Cherkin of Don Baxter and Co., Glendale, Calif.

[5] The presence of traces of alkali in the non-saponifiable residue presumably causes clouding of the $SbCl_3$ reagent. Also, it was found that the presence of a trace of petroleum ether would also prevent the satisfactory application of the Carr-Price method. Our procedures satisfactorily cleared up these difficulties.

In order to determine whether the administration of the supplementary vitamin A was accompanied by changes in other constituents of the milk, estimations were also made on protein, fat, ash and total solids. Protein was determined by the usual macro-Kjeldahl procedure, fat by the Babcock method and total solids and ash by the usual methods.

RESULTS

The results on vitamin A and carotene are summarized in table 1.

The vitamin A content in the non-supplemented group averaged 331 I.U. as preformed vitamin A per 100 ml. or 424 I.U. when the activity of the carotene is included during the period 2 to 10 days following parturition but not including samples of colostrum. In the later periods the total vitamin A averaged about 270 I.U. per 100 ml. of milk. The averages of Dann ('32) made at a number of periods during lactation are intermediate between these although this investigator reports an average value of total vitamin A of 632 I.U. per 100 ml. for human colostrum (111 samples).

In general, progressively higher values were obtained with the increasing doses of vitamin A, the values for the 2- to 10-day period being 599, 869, and 1047 I.U. for the 50,000, 100,000, and 200,000 I.U. supplement groups, respectively. In all cases these values are statistically higher than that for the control. In general, there is a decrease in vitamin A parallel to that in the control group in the later periods with all levels of vitamin A intake. In most of the cases in groups 3 and 4, the differences are marked and are statistically significant where there were enough observations for such statistical treatment. However, in group 2 the values although considerably higher than the controls are statistically significant only in the first period.

The maximum value for vitamin A of 2160 I.U. per 100 ml. was found in milk obtained during the 2- to 10-day period from a patient in group 4. Values almost as high (1992 and 1746) were obtained in the milk of patients in group 3.

Even though in all groups the quantities of vitamin A in the milk tend to fall in the 11- to 30- and 31- to 60-day periods as compared with that found in the 2- to 10-day period, they are somewhat higher in the last period. There is no evidence, therefore, that the continued adminis-

TABLE 1

The vitamin A and carotene in human milk of subjects who had received supplementary vitamin A from the sixth month of pregnancy and of controls who had received no supplementary vitamin A.

PERIOD POST-PARTUM	VITAMIN A IN I.U.[1] IN GROUP				CAROTENE IN MICROGRAMS [1] IN GROUP				TOTAL VITAMIN A IN I.U. IN GROUP			
	1: Control	2: 50,000 I.U.	3: 100,000 I.U.	4: 200,000 I.U.	1	2	3	4	1	2	3	4
days 2–10	331 ± 21 (40)	599 ± 87 **3.01** (16)	869 ± 121 **3.90** (15)	1047 ± 181 **3.93** (11)	56 ± 5	89 ± 12	101 ± 21	78 ± 15	424	747	1037	1177
11–30	232 ± 25 (8)	471 ± 114 **2.05** (8)	498 ± 96 **2.67** (8)	871 ± 123 **4.22** (8)	43 ± 3	34 ±5	38 ± 6	32 ± 6	304	528	561	928
31–60	171 ± 21 (13)	325 ± 117 **1.29** (6)	459 ± 74 **3.75** (8)	501 [2] (5)	38 ± 7	24 ± 6	40 ± 6	30 [2]	234	365	526	551
61 +	216 ± 30 (24)	289 ± 14 **2.15** (16)	795 ± 121 **4.66** (15)	660 [2] (2)	39 ± 6	33 ± 4	54 ± 9	35 [2]	281	344	885	718

The figures in parentheses are the number of observations in the average. The numbers in average are similar in corresponding groups in carotene and total vitamin A columns.

The bold face figures are the ratio of mean difference to standard error of mean difference when compared with control group. When this exceeds 3.0, the results are considered significant.

[1] Including the probable error of the mean calculated as follows: $\sqrt{\frac{\Sigma d^2}{n}} / \sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

[2] Too few observations for a statistical evaluation.

tration of the large doses of vitamin A results in its greater destruction with a lowered amount being secreted in the milk. For example, the vitamin A excretion remained high in subject AF over a period of 6 months as shown by the following analyses for vitamin A in I.U. per 100 ml. of milk: 1452 on the 6th day, 840 on the 42nd, 1296 on the 73rd, 1392 on the 90th, 1746 on the 135th and 1032 on the 180th day.

TABLE 2

The average total solids, protein, fat and ash in the milk of women receiving no supplementary vitamin A (group 1), 50,000 I.U. (group 2), 100,000 I.U. (group 3) or 200,000 I.U. (group 4) daily at various intervals during lactation.

GROUP AND SAMPLE	SAMPLE NO.	NO. OF SAMPLES[1]	TOTAL SOLIDS	PROTEIN	FAT	ASH
			%	%	%	%
Group 1 Control	1	40	12.15 ± 0.13(a)	1.81 ± 0.11(e)	2.7 ± 0.4	0.304 ± 0.026(a)
	2	8	12.58 ± 0.40(a)	1.50 ± 0.13(a)	3.5 ± 0.6	0.233 ± 0.036(a)
	3	13	11.44 ± 0.50	1.30 ± 0.15	2.5 ± 0.5	0.175 ± 0.063
	4	24	12.27 ± 0.45(a)	1.27 ± 0.04(a)	3.8 ± 0.4	0.209 ± 0.050(a)
Group 2 50,000 I.U.	1	15	11.59 ± 0.21	2.10 ± 0.22	2.4 ± 0.3	0.330 ± 0.082
	2	8	11.92 ± 0.46(a)	1.47 ± 0.15	3.3 ± 0.4	0.252 ± 0.023(a)
	3	5	12.20 ± 0.64	1.40 ± 0.01	3.1 ± 0.5	0.254 ± 0.072
	4	16	11.50 ± 0.29(c)	1.10 ± 0.09(c)	3.0 ± 0.4	0.211 ± 0.011(c)
Group 3 100,000 I.U.	1	14	11.32 ± 0.58(a)	2.05 ± 0.14	3.1 ± 0.3	0.313 ± 0.015(a)
	2	8	12.69 ± 0.39(a)	1.54 ± 0.56	3.3 ± 0.3	0.265 ± 0.019(a)
	3	8	12.40 ± 0.45	1.39 ± 0.04	4.3 ± 0.7	0.224 ± 0.009
	4	15	14.36 ± 0.82(a)	1.19 ± 0.12	5.5 ± 0.7	0.198 ± 0.050(a)
Group 4 200,000 I.U.	1	11	12.13 ± 0.32(a)	2.17 ± 0.19(a)	2.9 ± 0.4	0.325 ± 0.019(a)
	2	8	12.72 ± 0.57	1.59 ± 0.13	3.9 ± 0.5	0.238 ± 0.045
	3[2]	5	12.57	1.58	3.9	0.288
	4[2]	2	12.48	1.40	4.4	0.205

Sample 1, 2–10 days after parturition: sample 2, 11–30 days; sample 3, 31–60 days; sample 4, 61 days plus.

[1] Number of fat samples. When the number of determinations considered in calculating the average was 1 less, they are marked with "a"; 2 less "b"; 3 less "c"; 5 less "e".

[2] Too small number for statistical evaluation.

There is also a marked tendency for milk carotene to reach considerably lower levels in the periods following the one immediately after parturition. There are no consistent differences between the various groups with respect to milk carotene.

Table 2 gives a summary of the total solids, protein, fat and ash content of the various samples.

No differences were observed in the protein or ash contents obtained at comparable periods of lactation irrespective of whether the subjects were receiving vitamin A or not. There is a progressive drop in protein as lactation progresses as was noted by Holt, Courtney and Fales ('15) as well as by Bell ('28); a similar decrease in ash content was found as reported by the former investigators. On the other hand, there would appear to be a slightly higher fat content in groups 3 and 4 during the last two periods when compared with the control group. In general the fat content steadily increases as lactation progresses, as proved by Bell ('28).

DISCUSSION

The administration of vitamin A in large doses effectively increases the vitamin A content in the milk of the lactating human subject in the same way as in the cow. Although a statistically significant rise was observed in the early milk after the administration of 50,000 I.U. of vitamin A daily, the increase was not maintained in the later samples. On the other hand, the administration of 100,000 I.U. of this vitamin daily more than doubled the vitamin A content of the milk and this augmentation was maintained for at least 6 months which was the maximum length of time that the studies were continued. The failure of previous workers (McCosh, Macy, Hunscher, Erickson and Donelson, '34; Dann, '36) to demonstrate this rise in vitamin A content of milk after ingestion of moderate amounts of vitamin A is readily explainable because the level of the dose administered was too low. As we have shown earlier with the cow (Deuel et al., '41), there is a considerable range over which no increase in vitamin A occurs. Thus, no increase was noted until 700,000 I.U. per day were fed; at levels of 235,000 and 470,000 I.U. daily the results were completely negative. In the human the threshold would appear to be in the neighborhood of 50,000 I.U. daily. As the quantity administered is increased above this amount, the quantity excreted is also gradually increased.

In order to test how quickly the women would respond to vitamin A therapy, a supplement of 200,000 I.U. was given daily to nine women who were 3–7 months post-partum and who had previously received no supplementary vitamin A. The average value of vitamin A in the milk 7 to 10 days after the vitamin A was started was found to be 452 I.U. per 100 ml. which is almost double the control value of 216 I.U. In one case (L.), the rise was from 27 to 774 I.U.

There is no clear-cut demonstration of a decrease in content of milk carotene associated with the increased vitamin A ingestion similar to the effect previously found in the cow (Deuel et al., '41; '42) and in the eggs of chickens (Deuel et al., '43). This may be an example of a species difference which is quite widespread in the metabolism of the carotenoids (Zechmeister, '37). On the other hand, it was impossible to control the diets in the case of the experiments on human subjects, and it it is conceivable that any depressing effect on carotene excretion which otherwise might have been observed may have been obscured and counteracted by the variations in the quantity of carotene ingested.

The administration of the large doses of vitamin A was not attended by any marked alterations in other components of the milk. Thus, the deleterious effects exhibited by a decreased fat content of the milk noted by Goldring et al. ('26) and others in the cow after the administration of large amounts of cod-liver oil were absent here, as we have noted earlier when shark-liver oil and vitamin A concentrates were given to cows. In the present tests, there is not only no evidence of a decreased content of fat, but there is an increase in most instances where the vitamin A is administered although this was not found to be a statistically significant rise. Also, there is apparently no alteration in the proportion of other components in the milk. There is a progressive lowering in protein and ash and a rise in fat content as the lactation cycle proceeds in the confirmation of earlier published results of other workers.

There is no evidence of any deleterious effects of the vitamin A on the mothers or offspring. The babies without exception grew well. Although in most cases, lactation was voluntarily terminated in several months, in one case it was continued satisfactorily for 6 months and the milk production was not diminished over what would be expected in women receiving no supplementary vitamin A. We have, however, failed to confirm the report of Straumfjord ('40) on the effect in decreasing vernix caseosa, although this may be explained by the fact that the period of vitamin A supplementation was limited only to the last trimester rather than the last two trimesters of pregnancy.

SUMMARY

1. The average amount of vitamin A in the milk of women receiving no supplementary vitamin A was 331 I.U. per 100 ml. for the 2- to 10-day period, 232 I.U. for the samples collected between the eleventh and thirtieth days, 171 I.U. for the 31- to 60-day samples and 216 I.U. for the samples collected after 2 months.

2. The administration of vitamin A in amounts of 50,000 I.U. daily starting with the sixth month of pregnancy caused a statistically sig-

nificant increase in the early milk but the significant difference was not maintained in the later periods; after 100,000 I.U. the vitamin A was doubled and the increase was obtained throughout. After the ingestion of 200,000 I.U. per day the values were over three times the level in the unsupplemented group.

3. The maximum level obtained was 2160 I.U. per 100 ml. during the 2- to 10-day period following parturition in the group receiving 200,000 I.U. daily.

4. No changes were noted in protein, fat or ash content or total solids by supplementation with vitamin A. Protein and ash decreased progressively and fat increased as the lactation cycle proceeded.

5. No depression in carotene excretion similar to that previously noted in cows and in chickens concomitantly with the administration of large doses of vitamin A was noted in the present tests.

6. There is no evidence of a deleterious effect caused by the continued administration of the large dosages of vitamin A, employed in the present tests, to women during the last trimester of pregnancy and during the lactation period. On the other hand, these data should not be interpreted to mean that the feeding of large doses of vitamin A to pregnant and lactating women is either necessary or desirable.

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THE RELATIONSHIP OF GLYCERIDE STRUCTURE TO FAT DIGESTIBILITY

I. SYNTHETIC GLYCERIDES OF STEARIC AND OLEIC ACIDS [1]

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INTRODUCTION

In recent years considerable research has been directed toward the study of fat digestibility. Most of the work has been focused on the relative coefficients of digestibility of various natural and hydrogenated fats and oils that are used in edible products. The ultimate purpose of such research should be, and in the main has been, a better knowledge of the fundamental principles of fat digestion and metabolism.

There is, among others, one basic question as yet unanswered. What is the single factor, or combination of factors, which limits the digestibility of certain fats? In 1915, Langworthy and Holmes, in a study with human subjects, concluded that "of those fats tested, the fats of low melting point are capable of more complete assimilation than those of high melting point". From later work Lyman ('17) decided that the melting point was not the only, and probably not the chief factor determining the rate of hydrolysis and absorption. Hoagland and Snider ('42) were unable to find a consistent relationship between melting point and digestive coefficient. Nor were they able to correlate digestibility with per cent of saturated acids in the fat or with linoleic acid content. These results have been corroborated by results obtained at the University of Pittsburgh (Longenecker et al., '44).

There have been, however, several instances where stearic acid and its esters have been found to be poorly digested. As early as 1890 Arnschink found tristearin only 9–14% digestible in dogs, with nearly all the undigested stearin being found in the feces as triglyceride. On the other hand, Lyman ('17) found that dogs utilized 95% of the tripalmitin and 82% of the palmitic acid that were present in their diets. Hoagland and Snider ('43) also found tristearin and stearic acid to be poorly absorbed, while tripalmitin and palmitic acid were much more digestible.

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[1] Presented before the Division of Biological Chemistry of the American Chemical Society in New York City, September, 1914.

It would appear, then, that further elucidation of the limiting factors of digestibility of fats would result from additional experiments with synthetic glycerides and mixtures of glycerides. In the present communication digestibility studies have been conducted with diets containing synthetic mono-oleodistearin, mono-stearodiolein, and 2:1 and 1:2 mixtures of triolein and tristearin.

EXPERIMENTAL

Preparation of glycerides

Oleic acid was prepared by fractional crystallization of the methyl esters of red oil,[2] according to the method outlined by Brown ('41), followed by saponification and acidification of the purified methyl oleate. The acid chloride was prepared by the reaction of oleic acid with oxalyl chloride (Wood et al., '44), followed by fractional distillation. Stearoyl chloride was obtained by refluxing stearic acid[3] with thionyl chloride, and distillation of the resulting product. Both acid chlorides were water white.

The simple triglycerides were synthesized by direct esterification of the fat acids with equivalent amounts of dry glycerol, with about 1% of p-toluene sulfonic acid present as catalyst (Wheeler et al., '40). The reactions were carried out at temperatures of 120–130°C. for triolein and 145–150°C. for tristearin, with a constant stream of dry nitrogen bubbling through the reaction mixtures. The triolein was purified by washing it in ether solution twice with 70% ethanol containing KOH and five times with 70% ethanol (Wheeler et al., '40). Tristearin was purified by crystallization from 95% ethanol.

Mono-olein and mono-stearin were prepared by the acetone-glycerol method (Malkin and Shurbagy, '36). To redistilled acetone-glycerol in solution in a dry quinoline and chloroform mixture were added slowly, with shaking and cooling, the respective acid chlorides. The reaction was permitted to proceed at room temperature for about 36 hours, and then the mixture was taken up in ether, washed, dried, and distilled to remove the solvent. The acetone complex was decomposed by cold HCl. The resultant mono-stearin was filtered and the precipitate was washed thoroughly with ice water. The mono-olein was taken up in ether for washing.

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[2] Red oil, which is expressed from tallow fatty acids, contains 80% or more oleic acid.

[3] Eastman Kodak Co., C.P. Grade.

Mono-oleodistearin was obtained by the reaction of mono-olein with stearoyl chloride in solution in dry quinoline and chloroform. The reaction mixture was kept warm on a steam bath for 4 hours, then cooled and taken up in ether. The ether solution was washed with 0.5 N H_2SO_4 and with 5% K_2CO_3 solution, and then was dried and distilled to remove the larger portion of the solvent. Ethanol was added until there was a slight cloudiness and the glyceride was crystallized from solution at – 22°C., and dried in a vacuum desiccator. Mono-stearodiolein was prepared in a similar fashion from mono-stearin and oleoyl chloride.

Composition of diets

The basic diet used was as follows: crude casein, 18%; experimental fat, 15%; salt mixture, 7%; liver extract concentrate, 3%; brewers' type yeast, 1%; and dextrose, 56%. Four diets were prepared, differing only in the composition of the experimental fat: Diet A, 2 parts tristearin, 1 part triolein; diet B, 1 part tristearin, 2 parts triolein; diet C, distearo-mono-olein; and diet D, mono-stearodiolein. For the low fat diet, the above ratio of components was used, with no fat added.

Feeding methods and collection of feces

A group of five animals was started on each of the four diets. A 1-day orientation period was allowed on each diet, followed by an 8-day experimental period during which food consumption was measured and feces were collected. The feces were stored in methanol until analysed. At the end of the 8 days the animals were changed to a stock diet for 2 days, and then put back on the experimental diets again.

For the second experimental period the animals which had been on diet A were given diet C, and vice versa. The same was done with diets B and D. In this way it was possible to check each diet with two different groups of animals, and to compare the reaction of each group to two different types of glycerides (mixed glycerides vs. mixture of simple triglycerides) containing the same proportion of stearic and oleic acids. Unfortunately, we were able to continue the second period only so long as our diets lasted, which varied from 2 to 6 days. The data showing food consumption and fecal elimination are presented in table 1.

Extraction of fecal lipid

Total lipids were extracted and digestibility coefficients were calculated in approximately the same manner as was employed by Hoagland and Snider ('42). The feces were saponified in the methanol in which they had been stored after mashing the fecal pellets. The saponification mixture was acidified with 35% H_2SO_4 and then extracted thoroughly with ether. The extracts were washed free of mineral acids with water, dried, and then the solvent was removed. The residues were evacuated at steam bath temperature until constant weight was attained.

From the weight of the acidic residue obtained in each case was subtracted the corresponding amount of lipid obtained on the low fat diet. The difference was multiplied by the factor 1.045 to convert to glyceride weight. The digestibility coefficients were determined from the calculated weight of excreted glyceride and the total amount of experimental fat ingested. These data are listed in table 1.

TABLE 1

Fat ingestion and excretion of rats on diets containing synthetic glycerides of stearic and oleic acids.

	EXPERIMENTAL DIETS								
	Low fat	*A*	*A*	*B*	*B*	*C*	*C*	*D*	*D*
Number of animal days	70	32	30	40	15	40	12	40	8
Food consumption (gm.)	..	340	528	583	239	628	163	596	97
Food per animal per day	..	10.6	17.6	14.6	15.9	15.7	13.6	14.9	12.1
Wt. fat in food (gm.)	..	51.0	79.2	87.5	35.9	94.2	24.5	89.4	14.6
Wt. feces (gm.)	124.1	91.4	124.1	113.5	40.4	119.8	25.6	114.7	15.4
Wt. acidic extract (gm.)	2.5	29.3	48.4	27.6	11.3	39.4	9.0	24.7	4.0
Corrected for low fat diet		28.2	47.3	26.2	10.8	38.0	8.6	23.3	3.7
Corrected for low fat diet × 1.045		29.5	49.4	27.4	11.3	39.7	9.0	24.3	3.9
Per cent of total fat undigested		57.8	62.4	31.3	31.5	42.1	36.7	27.2	26.7
Digestibility (%)		42.2	37.6	68.7	68.5	57.9	63.3	72.8	73.3
Digestibility based on combined periods		39.4		68.6		59.0		72.9	

TABLE 2

Analysis of lipid extracted from feces of rats on diet containing synthetic glycerides.

	EXPERIMENTAL DIET			
	A	*B*	*C*	*D*
Iodine value	12.3	23.8	18.0	28.5
Acid number	188.5	182.5	172.5	145.0
Wt. % palmitic	2.1	2.6	0.5	...
Wt. % stearic	81.8	67.8	78.2	65.8
Wt. % oleic	7.9	16.1	9.2	15.8
Wt. % non-saponifiable[1]	8.2	13.5	12.1	18.4

[1] Consists chiefly of distillation residue and material washed from column following distillation. Too dark for accurate saponification number, but probably contains up to 50% stearic acid.

Analysis of fecal lipid

The acidic fecal extracts from the two experimental periods were combined by groups and the iodine values and acid numbers were determined (table 2). Each combined extract was methylated and the mixed methyl esters were analyzed by the fractional distillation method for fat acid composition (table 2).

DISCUSSION

It is apparent from table 1 that there was very satisfactory agreement between the digestibility coefficients that were obtained during the first experimental period and the corresponding values for the second period. This agreement adds significance to the differences obtained between the digestibilities of the various experimental fats.

The results indicate rather clearly that stearic acid is very poorly digested, whether fed as a mixed glyceride or a simple triglyceride. Tristearin quite apparently is almost completely indigestible when mixed with triolein. When present in mixed glycerides, stearic acid becomes more indigestible as the amount of oleic acid is increased.

Inasmuch as it has been shown in previous work (Longenecker and Mattil, '42) that tristearin is partially utilized by the rat when it is the only fat in the diet, one is led to the thought that a degree of selective utilization may exist. That is, when tristearin is the sole fat in the diet, the rat is able to digest and metabolize stearic acid in order to fulfill partially, or perhaps completely, his energy requirements. When, however, a more desirable glyceride such as triolein is fed along with tristearin, the rat will attempt to meet his needs without stearic acid. Thus, it may be concluded that if the diet contained one of the customary edible fats, essentially all of the tristearin and most of the other stearic acid in the fat would not be metabolized, provided that the dietary requirements of the rat were met by the other more digestible fat acids.

The "solvent" action of oleic acid for stearic acid which tends to increase the digestibility of the latter is markedly greater with the mixed glycerides than with the mixtures of triglycerides. This may be due to the greater solubility of the mixed glycerides, as compared with tristearin, thus resulting in an increased rate of hydrolysis.

Consideration of these observations and of the ratios of stearic to oleic acids in the feces would seem to indicate that hydrolysis, or perhaps ester interchange, must precede absorption of glycerides as the general rule. If the fats were absorbed or rejected chiefly as the original triglycerides, one would expect to find approximately the same stearic to oleic ratio in the feces as in the food in groups C and D. Such, however, is far from the case (table 2). The only alternative hypothesis which lends itself would be the complicated absorption → hydrolysis → resorption system, which seems the less probable of the two.

It is interesting to note that the amounts of oleic acid in the fecal lipids are in direct proportion to the amounts in the ingested fat (table 2). The explanation for this is not readily apparent, although it is more probably due to mechanical or physical factors rather than chemical or metabolic.

SUMMARY

Synthetic glycerides containing stearic and oleic acids have been prepared and incorporated into the diets of rats. The stearic acid in the glycerides has been shown to be very indigestible. It is better utilized when fed as mixed glycerides than when fed as tristearin mixed with triolein. The possibility of selective utilization of fat acids has been indicated. Support is given to the hypothesis that either hydrolysis of glycerides or ester interchange precedes absorption.

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STUDIES ON BONE FRACTURE HEALING

I. EFFECT OF VITAMINS A AND D [1]

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Interest in the relation between the healing of fractures and diet has been intensified by the war not only because of the increased incidence in the armed forces but also as a result of the dietary deficiencies of the civilian population. It is also a serious problem in those of advancing age for not only is delayed fracture healing more common in this group, but their diets are frequently lacking in essential food elements.

Excellent histological studies (Hertz, '36; Urist and McLean, '41) have demonstrated the effects of deficiencies of vitamins A, C, and D on the fundamental cellular processes involved in fracture healing. However, these studies are of necessity only qualitative.

The present investigation was undertaken to obtain quantitative data on the activity of calcification and the functional recovery in standard fractures of the fibulae of animals with experimentally produced dietary deficiencies. It was presumed that such quantitative methods might provide a means for detecting the effects of mild dietary deficiencies and so indicate the optimum level for fracture healing. They would also serve to test the effect of other measures designed to promote or delay fracture healing.

EXPERIMENTAL METHODS

Fractures of the middle of the right fibula (opposite the tibial spine) were produced in rats by the fracture forceps of Hertz ('36) or by operation. The fibula bears no weight, and is splinted naturally by the tibia so that no further care of the fracture is necessary. Animals were sacrificed at 4-day intervals over a period of 3 or 4 weeks. Twenty-four hours before each animal was sacrificed, it was given a dose of 0.2 mg. Sr* [2] as the lactate by intraperitoneal injection. The Sr* taken up by the fractured fibula was measured directly with a Geiger-Müller counter, and compared with that taken up by the unfractured bone on the opposite side. The excess Sr* in the fractured bone was taken as a measure of the calcification activity in the callus. This was expressed by the formula:

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[2] The symbol Sr* designates strontium "labeled" by the presence of its radioactive isotope.

$$\text{Calcification Activity} = (\%\ \text{dose Sr}^*\ \text{in fractured bone} - \%\ \text{dose Sr}^*\ \text{in normal bone}) \times 10$$

Radio-autographs of fractured and control bones showed that the excess Sr* in the fractured bone was localized in the callus. The radio-autographs were prepared by mounting the fractured and normal fibulae from typical animals on a rubber block in direct contact with No-Screen x-ray film. The radiations from the Sr* produced a darkened image of the bone corresponding to the sites of active deposition.

The functional recovery was determined by measuring the breaking strength of both the fractured fibula, and of the unfractured bone on the opposite side. The apparatus used was a modification of that of Lindsay and Howes ('31) and was patterned after the standard engineering device for measuring the strength of beams. The bone is supported on the balance pan of a 1 kg. Toledo scale by two knife edges spaced 10 mm. apart. Pressure is exerted from above by two knife edges spaced equidistant from each other and from the supports. These are pivoted to adapt to any irregularity of the bone. In this way, the stress remains uniform between these points, over the central portion of the bone, including the site of the fracture. Pressure is increased at a uniform rate by rotation of a micrometer screw which presses down the upper knife edges. The actual load on the bone is read directly on the balance scale, and the load at which the bone breaks is taken as the breaking strength.

The functional recovery during healing is indicated by the increase in the breaking strength of the fractured bone, while the relative strength of the fracture as compared to the normal bone may be expressed by the ratio of the two breaking strengths; i.e.,

$$\text{Relative Fracture Strength} = \frac{\text{Breaking strength of fractured fibula}}{\text{Breaking strength of the unfractured fibula}}$$

RESULTS

Normal rats on stock diet

The rats used in this series were young adult animals 2 to 3 months old which had been reared and maintained on the standard stock colony diet. The results obtained from over ninety animals are summarized in table 1. The figures given are the average values plus or minus the standard deviation.

In these normal animals, the calcification activity was very pronounced during the period from 8 to 16 days after the bone was fractured, corresponding to the primary calcification of the callus. The breaking strength of the fractured bone, insignificant at 4 days while the callus was soft, rose rapidly until by 12 to 16 days its value was comparable to that of the unbroken bone on the opposite side. Apparently in the rat, the fractured bone quickly attains and maintains a strength comparable to that of the normal bone.

TABLE 1

Fracture healing in normal rats on stock diet.[1]

DAYS AFTER FRACTURE	NO. OF RATS	WEIGHT	CALCIFICATION ACTIVITY IN % DOSE OF SR* × 10			BREAKING STRENGTH		RELATIVE FRACTURE STRENGTH
			Fractured	Unfractured	"Callus"	Fractured	Unfractured	
		gm.				*kg.*	*kg.*	
4	7	175 ± 14	2.0 ± 0.5	1.5 ± 0.5	0.5 ± 0.3	0.00	0.53 ± 0.23	0.0
8	15	200 ± 26	3.7 ± 0.6	1.5 ± 0.5	2.2 ± 0.7	0.31 ± 0.13	0.55 ± 0.20	0.6
12	13	197 ± 25	5.4 ± 1.1	1.7 ± 0.4	3.7 ± 1.1	0.51 ± 0.12	0.63 ± 0.16	0.8
16	13	190 ± 27	4.1 ± 1.4	1.6 ± 0.4	2.5 ± 1.3	0.52 ± 0.14	0.58 ± 0.14	0.9
20	18	195 ± 24	3.0 ± 0.7	1.7 ± 0.5	1.3 ± 0.6	0.53 ± 0.18	0.67 ± 0.16	0.8
24	12	206 ± 21	2.9 ± 0.9	1.5 ± 0.7	1.4 ± 0.8	0.63 ± 0.18	0.75 ± 0.11	0.8
28	8	225 ± 40	2.5 ± 0.8	1.6 ± 0.7	0.9 ± 0.6	0.53 ± 0.21	0.66 ± 0.16	0.8
32	6	202 ± 32	2.5 ± 0.7	1.4 ± 0.2	1.1 ± 0.6	0.65 ± 0.20	0.68 ± 0.12	0.9

[1] Data given are mean values ± the standard deviation.

Effect of vitamin A deficiency

The observations of Mellanby ('41, '44), Wolbach and Bessey ('41), and Moore ('39) have established bone overgrowth as an important aspect of vitamin A deficiency. Mellanby believes that lack of vitamin A leads to increased activity of both the osteoblasts and osteoclasts of the bone with proliferation of cancellous bone at the expense of compact bone. Wolbach and Bessey, on the contrary, consider that vitamin A deficiency retards bone growth. Hertz ('36) observed that cartilage formation in the callus was inhibited in vitamin A deficient rats, although the fracture ossified and consolidated at the same time as in normal rats.

In view of these observations, and because of the frequent deficiency of vitamin A, particularly in war dietaries, fracture studies were carried out on vitamin A deficient rats both with and without vitamin A supplements. Rats were weaned at 3 weeks to the standard vitamin A test diet (U. S. P. XI) and maintained on this diet for 5 weeks.[3] After the bones were fractured, the rats were divided into two groups, both of which were continued on the vitamin A deficient diet. The treated group received 10,000 units of vitamin A in shark liver oil at the time of fracture and at weekly intervals thereafter, while the untreated group received no supplement. The mortality among the untreated rats was high, and so the process of healing could not be followed beyond 16 days. The results obtained are summarized in table 2.

TABLE 2

Effect of vitamin A on fracture healing.[1]

DAYS AFTER FRACTURE	NO. OF RATS	WEIGHT	CALCIFICATION ACTIVITY IN % DOSE OF SR* × 10			BREAKING STRENGTH		RELATIVE FRACTURE STRENGTH
			Fractured	Unfractured	"Callus"	Fractured	Unfractured	
		gm.				*kg.*	*kg.*	
			Vitamin A deficient diet					
8	14	146 ± 18	3.0 ± 0.7	1.7 ± 0.4	1.3 ± 0.5	0.24 ± 0.10	0.62 ± 0.09	0.4
12	11	140 ± 26	2.3 ± 0.7	1.4 ± 0.4	0.9 ± 0.5	0.30 ± 0.08	0.72 ± 0.11	0.4
16	12	129 ± 18	2.7 ± 0.6	1.5 ± 0.4	1.2 ± 0.4	0.44 ± 0.15	0.63 ± 0.11	0.7
			Vitamin A deficient diet plus 10,000 units of A per week					
8	11	168 ± 24	4.3 ± 0.6	2.7 ± 0.4	1.5 ± 0.8	0.41 ± 0.19	0.73 ± 0.12	0.6
12	10	167 ± 10	3.7 ± 0.7	2.1 ± 0.3	1.6 ± 0.6	0.54 ± 0.18	0.74 ± 0.10	0.7
16	10	161 ± 40	3.8 ± 0.6	2.4 ± 0.3	1.4 ± 0.6	0.54 ± 0.12	0.71 ± 0.19	0.8
20	6	174 ± 16	3.2 ± 0.6	1.8 ± 0.4	1.4 ± 0.3	0.59 ± 0.07	0.79 ± 0.11	0.8

[1] Data given are mean values ± the standard deviation.

The fracture callus of both treated and untreated groups was considerably smaller than that of the typical callus, and the "calcification activity" was less. However, the fracture strength of the treated rats, as indicated by the breaking strength, recovered just as quickly as it did in the control animals on stock diet. On the other hand, the recovery in breaking strength was significantly delayed in the untreated vitamin A deficient animals. It may be that this delay in fracture healing was occasioned by the general debility of these animals rather than any specific effect of the vitamin deficiency. It may be noted that no significant effect of vitamin A deficiency on the distribution and excretion of Sr* was observed by us in tracer experiments.

Effect of vitamin D deficiency

The importance of vitamin D in the mineralization of bone has been established by numerous investigators. The healing process in the fracture callus of rachitic rats has been studied by a number of workers using histological methods (Hertz, '36; Compere, Hamilton and Dewar, '39; Urist and McLean, '41). These investigators found that rickets exerted a profound effect on the formation and mineralization of the fracture callus.

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[3] We are indebted to Mr. Mittler of the Laboratories of the California Packing Corporation for rearing the vitamin A deficient rats used in this investigation.

The effect of rickets was studied with our techniques in the following experiments. Rats were weaned at 24 days to the Rachitogenic Diet no. 2 (U.S.P. XII) and depleted for 25 days according to the usual technique.[4] It was necessary to produce the fractures of the fibula by operation because the bones were too soft for effective use of the fracture forceps. The animals were divided into two groups, both of which were maintained on the rachitogenic diet. One group received a supplement of 10 units of vitamin D (as irradiated ergosterol) per day, while the other group received no treatment. The results obtained are summarized in table 3.

The fibulae of all the rats were thin, poorly calcified, and pliable, so that it was difficult to obtain a clear-cut breaking strength measurement. The callus was large, soft, and cartilaginous. In the animals with severe rickets no appreciable deposition of Sr* in the callus was evident either by direct measurement, or in radio-autographs. The breaking strength of the fractured bone was ill-defined due to the pliability of the bone, and while comparable to that of the unbroken bone on the opposite side, it was much less than that of the fibula of normal rats of the same age.

In those rachitic rats which received treatment some calcification did take place in the callus, but the calcification activity was much less than in normal rats, and the breaking strength resembled that of the untreated rachitic animals.

Effect of hypervitaminosis D

Because of the marked effect of toxic doses of vitamin D on calcium metabolism, and the frequent use of large doses of vitamin D in therapy, it was felt that a study of its effect on fracture healing might be of value. Grauer ('32) found that overdosage of vitamin D produces a stimulation of the fibrous layer of the periosteum through decalcification of the bone. Consequently there is a retardation of bone repair.

Young adult female rats 2 or 3 months old were used in the experiments. They were reared and maintained on the regular stock colony diet. After fracture had been produced in the usual way, they were given 40,000 units of vitamin D (as irradiated ergosterol) by mouth

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[4] We are indebted to Mr. Theodore Sanford of the Booth Laboratories, Emeryville, for rearing the rachitic rats used in these investigations.

TABLE 3

Effect of vitamin D on fracture healing.[1]

GROUP	DAYS AFTER FRACTURE	NO. OF RATS	WEIGHT	CALCIFICATION ACTIVITY IN % DOSE OF SR* × 10			BREAKING STRENGTH		RELATIVE FRACTURE STRENGTH
				Fractured	Unfractured	"Callus"	Fractured	Unfractured	
			gm.				*kg.*	*kg.*	
Severe rickets									
Ricketogenic	8	3	78 ± 6	0.7 ± 0.4	0.7 ± 0.5	0.0 ± 0.2	0.10 ± 0.02	0.17 ± 0.02	0.6
diet;	12	4	103 ± 2	0.7 ± 0.2	0.7 ± 0.3	0.0 ± 0.2	0.22 ± 0.08	0.23 ± 0.01	1.0
no supple-	16	2	87 ± 10	0.3 ± 0.1	0.5 ± 0.2	— 0.2 ± 0.1	0.19 ± 0.02	0.21 ± 0.05	0.9
ment	20	5	103 ± 18	0.4 ± 0.2	0.5 ± 0.2	— 0.1 ± 0.1	0.16 ± 0.07	0.22 ± 0.06	0.7
Treated rickets									
Ricketogenic	8	4	83 ± 21	1.6 ± 0.4	1.0 ± 0.3	0.6 ± 0.6	0.13 ± 0.01	0.16 ± 0.03	0.8
diet plus	12	3	72 ± 3	2.2 ± 0.5	1.4 ± 0.3	0.8 ± 0.5	0.19 ± 0.03	0.18 ± 0.03	1.1
10 units	16	4	98 ± 15	1.7 ± 0.5	0.9 ± 0.1	0.8 ± 0.5	0.20 ± 0.08	0.20 ± 0.09	1.0
vitamin D per day	20	4	85 ± 19	1.0 ± 0.1	0.7 ± 0.2	0.3 ± 0.2	0.15 ± 0.08	0.18 ± 0.05	0.8
Hypervitaminosis D									
Stock diet	8	5	168 ± 20	2.0 ± 0.4	0.8 ± 0.1	1.2 ± 0.4	0.12 ± 0.04	0.54 ± 0.05	0.2
plus 10,000	12	4	176 ± 23	1.4 ± 0.1	0.6 ± 0.2	0.8 ± 0.3	0.35 ± 0.16	0.68 ± 0.13	0.5
units vitamin D	16	3	143 ± 5	1.4 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	0.26 ± 0.04	0.50 ± 0.04	0.5
per day	20	3	173 ± 11	1.6 ± 0.3	0.8 ± 0.2	0.9 ± 0.3	0.29 ± 0.06	0.51 ± 0.06	0.6

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[1] Data given are mean values ± the standard deviation.

every 4 days. The animals showed no toxic signs other than a loss of 5 to 10 gm. in weight per week. The results obtained are given in table 3.

In these rats, both the callus size and the calcification activity was much less than in the normal rats, and the recovery in breaking strength was greatly impaired.

SUMMARY

1. A method for studying healing in standard fractures of the rat fibula is described, in which calcification activity is determined by measuring the uptake of Sr^* by the callus, and functional recovery by the increase in breaking strength of the fractured bone.
2. In normal rats, the most active calcification in the callus occurs over the period from 8 to 16 days. The broken bone attains a strength comparable to that of the normal bone on the opposite side within 12 to 16 days.
3. In vitamin A deficient rats, the callus is smaller than in normal animals, and the calcification is less active. In those treated with large doses of the vitamin the increase in strength of the fractured bone was comparable to that in normal rats. On the other hand the untreated animals showed a significant delay in fracture healing. This may have been due to the debilitated condition of the latter.
4. In rachitic animals, there is no significant calcification of the callus, unless vitamin D is added to the diet.
5. In animals receiving toxic doses of vitamin D, the callus is small, calcification activity is reduced, and recovery in strength is delayed.

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THE EFFECT OF SOY FLOUR ON THE NUTRITIVE VALUE OF THE PROTEIN OF WHITE BREAD[1]

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ONE FIGURE

(Received for publication December 11, 1944)

Previous studies have shown that the addition of 15 or 20% of soy flour significantly improves the protein quality of bread, but the product is so different from white bread that it is not accepted by the average consumer. Since 5% of soy flour can be used without appreciably altering the appearance or flavor of white bread it seemed of interest to determine whether such an addition would improve the protein quality.

This paper reports such a study with rats, using bread made from enriched patent flour and containing 3% of whole milk solids. Data are also presented on the milk solid content of a variety of types of national brands of breads purchased on the open market.

Jones and Divine ('44) reported the results of supplementing patent flour with different amounts of soy flour. The gain per gram of protein consumed was 0.75 gm. for the patent flour; 1.38 gm., 2.16 gm., and 2.27 gm. when 5%, 10%, and 15%, respectively, of the soy flour was added. Johns and Finks ('21) found that rats fed wheat bread gained only 1.0 gm., whereas on wheat-soy bread they gained 1.5 gm. for each gram of protein consumed. Kon and Markuze ('31) showed a similar improvement in the protein quality of bread by the addition of soy flour. In rat growth studies, they found that the average gain per gram of protein consumed was 1.09 gm. for wheat bread and 1.62 gm. when soy flour was added.

Mitchell and Carman ('26) reported a biological value of 50 for patent flour. Klein et al. ('26) reported an average biological value of 42 for patent flour. Fairbanks ('38, '39) in comparing breads containing 0, 6 and 12% non-fat milk solids found that increasing the milk solids improved the palatability and the growth-promoting value of the bread when fed to rats. Henry et al. ('41) reported biological values of 44.7 for water bread, 47.6 for 2% milk bread, and 49.7 for 6% milk bread.

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Mitchell et al. ('43) and Light and Frey ('43) have shown that 6% dried skimmilk solids in bread results in growth equal to that produced by whole wheat bread. Murlin et al. ('41) observed that although white bread has a higher true digestibility by human subjects, its relative value may be lower than a peeled wheat or whole wheat bread. They reported the following digestibility and relative values: lean white bread, 99.4 and 75.3; peeled wheat bread, 94.9 and 77.9; whole wheat bread (with 5% non-fat milk solids) 92.8 and 77.8 respectively.

STUDIES WITH RATS

Growth studies have been carried out with rats fed diets containing white bread, white bread with 5% soy flour (flour made from the soybean, Glycine hispida), patent flour, and patent flour with 5% soy flour. The biological value of the protein of white bread and bread with 5% soy flour was also measured.

Weanling albino rats fed the diets shown in table 1 were paired according to sex, weight, and litter. Each group used in the growth studies consisted of five males and five females. The animals were weighed twice each week during the 6-week period. The food was weighed daily and the food intakes were equalized. Scattering of the diets was slight but was taken into consideration when it occurred. A rice polish concentrate and crystalline riboflavin supplied daily approximately 30 μg. thiamine, 400 μg. nicotinic acid, 30 μg. pyridoxine, 55 μg. pantothenic acid, 5.4 μg. filtrate factor, 40 μg. riboflavin. Two drops of cod liver oil

TABLE 1

Composition and protein contents of the diets.

INGREDIENT	DIET NUMBER 1	2	3	4
	%	%	%	%
Patent flour	....	....	77.52	73.64
Soy flour (full-fat)	....	....	....	3.88
White bread	74.40	....	....	....
Wheat-soy bread	....	66.66	....	....
Sucrose	7.60	15.34	4.48	5.48
Butterfat	5.00	5.00	5.00	5.00
Soybean oil	5.00	5.00	5.00	4.00
Salt mixture[1]	4.00	4.00	4.00	4.00
Cellophane	4.00	4.00	4.00	4.00
Protein content (N × 5.7)	9.2	9.2	9.1	10.3

[1] Osborne and Mendel.

were fed three times each week. Distilled water was kept before the animals at all times.

The dough for the two breads [2] included a basal mixture containing whole milk solids 19.8 gm., yeast 19.8 gm., sucrose 26.4 gm., salt 13.2 gm., shortening 13.2 gm., and water 396 ml. To these ingredients were added 660 gm. of wheat flour for the white bread, and 627 gm. wheat flour and 33 gm. full fat soy flour [3] for the wheat-soy bread mix. The white bread contained 13.5% protein and the wheat-soy bread 14.9% protein on a dry matter basis.

RESULTS

The starting weight, gains, food consumption, protein intakes and the protein efficiencies for the rats fed the bread diets are shown in table 2. The rats receiving the wheat-soy bread diet in nine cases out of ten gained more weight than those fed the white bread diet. The difference in weight gains of the rats fed these bread diets is highly significant, odds 999:1. The grams of gain per gram of protein consumed were 20% higher for the wheat-soy bread than for the white bread diet.

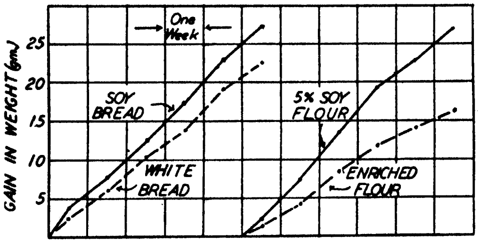

Fig. 1 Cumulative gain of rats fed white bread and white bread with 5% soy flour, and of those fed enriched patent flour with and without 5% soy flour.

Similar studies comparing the flours only (diets 3 and 4, table 2) gave average protein efficiencies of 0.80 gm. for white flour and 1.13 gm. for white flour plus 5% soy flour. Odds favoring significance of the difference between these average values were 4999:1.

A comparison of the progressive gains of the rats presented in figure 1, emphasizes the fact that the soy flour improved the growth promoting value of the diets and that the presence of 3% of whole milk solids did not mask that improvement. The difference between the diets became increasingly greater as the experiment progressed.

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[2] We acknowledge the assistance of Miss Catherine Personius, Professor of Home Economics, in making the breads.

[3] Sold by Soya Corporation of America, New York, N. Y.

Biological values. The biological values of the two breads were determined by the method of Mitchell ('43), using diets 1 and 2 (table 2) fed in the growth studies. During the basal period a low protein egg diet was fed. This diet contained 3.95% protein and was composed of the following ingredients: egg solids 7.8, cooked starch 69.2, sucrose 5.0, butterfat 5.0, soybean oil 5.0, O & M salt mix 4.0, and cellophane 4.0%.

Twelve male albino rats weighing 43 to 53 gm. were paired according to litter weight. The food intake of the pair-mates were equalized.

TABLE 2

Summary of the results of the growth study in which bread diets were fed.

RAT NO.	SEX	DIET [1]	STARTING WEIGHT	WEIGHT GAINS	FOOD INTAKE	PROTEIN INTAKE	GRAMS GAIN PER GRAM PROTEIN
			gm.	*gm.*	*gm.*	*gm.*	
21	Male	White bread	40	17	201	18.49	.92
31	Male	Wheat-soy bread	39	19	202	18.58	1.02
22	Male	White bread	44	26	265	24.38	1.07
32	Male	Wheat-soy bread	44	35	265	24.38	1.44
23	Male	White bread	47	24	255	23.46	1.02
33	Male	Wheat-soy bread	46	28	255	23.46	1.19
24	Male	White bread	45	16	207	19.04	.84
34	Male	Wheat-soy bread	47	16	207	19.04	.84
25	Male	White bread	54	22	282	25.94	.85
35	Male	Wheat-soy bread	56	26	283	26.04	1.00
26	Female	White bread	40	24	223	20.52	1.17
36	Female	Wheat-soy bread	39	29	223	20.52	1.41
27	Female	White bread	44	26	274	25.21	1.03
37	Female	Wheat-soy bread	42	28	274	25.21	1.11
28	Female	White bread	43	27	265	24.38	1.11
38	Female	Wheat-soy bread	43	31	265	24.38	1.27
29	Female	White bread	48	20	292	26.86	.74
39	Female	Wheat-soy bread	46	32	292	26.86	1.19
30	Female	White bread	50	27	289	26.59	1.02
40	Female	Wheat-soy bread	50	32	289	26.59	1.20
Average		White bread	45.5	22.9	255.3	23.49	.97
Average		Wheat-soy bread	45.2	27.6	255.5	23.51	1.17

[1] Both diets contained 9.2% protein.

The same vitamin supplements were given throughout this study as during the growth studies. A 6-day preliminary period of constant food intakes preceded each collection period. Collections of urine and feces were made for 7 days on each diet. The test diets were fed during the first period. The basal diet was fed during the second period. During the third period the test diets were reversed and again fed. The biological values obtained by this study are shown in table 3. The average value for wheat-soy bread is approximately 10% higher than the value for white bread. The mean difference between the biological values of these bread proteins is highly significant, odds 249 : 1.

TABLE 3

Biological value of the protein of white bread and soy bread.

BREAD	PAIR NUMBER						AVERAGE
	1	2	3	4	5	6	
White bread	41.9	38.4	49.2	41.8	41.9	49.8 [1]	43.3
Wheat-soy bread	45.4	49.4	48.3	48.2	46.0	56.4 [1]	47.7

[1] One urine sample was lost. Therefore, these represent a single value instead of an average of two values.

TABLE 4

Non-fat milk solids in bread purchased on the open market (dry basis).

TYPE OF BREAD	NUMBER OF SAMPLES	NON-FAT MILK SOLIDS	
		Range	Average
		%	%
White	11	1.83–4.29	3.07
White (milk solids)	8	0.35–6.20	2.78
Wheat breads [1]	8	0.34–2.49	1.30
Wheat breads (milk solids) [1]	6	0.56–3.07	1.62

[1] Includes breads labeled as wheat, cracked wheat, 75% whole wheat and 100% whole wheat.

Chemical analyses of breads for milk solids. Thirty-three samples of breads, mostly national brands were collected on the open market in July and August of 1942 and analyzed for milk solids. This was previous to the publication of Food Distribution Order no. 1 (Federal Register '42), requiring that white bread contain not less than 3 nor more than 4% of milk solids, which became effective January 18, 1943. The breads were obtained from the Ithaca, New York, market with the exception of four samples, two of which came from Kansas and two from Missouri. Milk solids were determined by the method of Magraw and Copeland ('36). The results are shown in table 4.

Only three of thirty-three breads examined contained as much as 6% of non-fat milk solids, although that level has frequently been quoted by bakers as being a desirable amount to include in white bread. The average non-fat milk solids for breads claiming milk as a constituent amounted to 2.28% of the dry bread, while the brands not claiming milk contained an average of 2.32%. The average for all brands was 2.30%, and approximately one-fifth of them contained less than 1%.

SUMMARY

Growth studies and biological values are reported which show that the addition of 5% soy flour significantly improves the growth promoting value of white bread which contains 3% of whole milk solids.

White bread containing 3% of milk solids gave 0.97 gm. gain per gram of protein consumed and bread with 5% soy flour, containing an equal amount of milk, 1.17 gm.; the difference is significant. The biological value of the protein of the white bread as determined by the Mitchell method was 43.3 as compared with 47.7 for the bread with 5% soy flour.

The analysis of thirty-three samples of breads purchased in 1942 showed a range from 0.34 to 6.20% in the content of milk solids (dry basis) and an average value of 2.30%. Breads labelled as containing milk varied as widely and contained no more milk solids than breads not so labelled.

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THE SULFUR BALANCE OF THE NON-LAYING, MOLTING AND LAYING HEN [1]

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ONE FIGURE

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Although sulfur in the reduced form is, like phosphorus and nitrogen, a normal constituent of the hen's egg, little or no attention has been given to the metabolism of this element in the hen or to requirements during laying and non-laying states. Supplements of cystine on a nitrogen-free diet were shown by Ackerson and Blish ('26) to have a sparing effect upon the endogenous nitrogen metabolism of the molting hen. A study of the loss or retention of sulfur on a laying ration, made up of commonly used ingredients, during periods of molting and egg production should give clues concerning the adequacy of the diet during each of these phases and possibly concerning any special needs for sulfur. The present investigation was planned to determine the sulfur balance of the hen during molting and during periods of low and high egg production.

EXPERIMENTAL

Four hens were used for the study. Each bird was housed in an individual cage with a mesh floor and fitted with a feeding cup designed for the measurement of feed consumption. The ration used consisted of 1 part ground wheat, 1 part ground corn, and 2 parts of a practical laying mash which had the following percentage composition: yellow corn 21, wheat middlings 20, wheat bran 20, meat scrap (55% protein) 15, pulverized oats 10, alfalfa meal 5, dried skimmed milk 5, calcite flour 2, salt 1 and cod liver oil 1.

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[1] Journal series paper of the New Jersey Agricultural Experiment Station, Department of Agricultural Biochemistry. From a thesis presented by Ralph T. Holman to the Graduate Faculty of Rutgers University in partial fulfillment of the requirements for the Master of Science degree.

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The excreta were collected on a glass plate, dried at room temperature with the aid of an electric fan, and ground in a Wiley mill. Charcoal or carmine was used as a marker in making the collections of excreta. Feathers were collected and clipped into fine pieces before analysis. A few feathers were shed during many of the collection periods, but only when the loss exceeded 0.5 gm. per day were the birds considered to be in a molt. Eggs were dried rapidly with a fan and ground in a mortar.

The oxygen bomb method of combustion used by Marston ('38) was chosen to bring about the destruction of organic matter and to convert reduced sulfur to the sulfate form. In early trials it was noted that a small quantity of incompletely burned material adhered to the nickel pan. This was prevented by lining the pan with ashless filter paper which served as an insulating material. The light bulky nature of feathers necessitated special precautions. After being clipped into small pieces, the weighed sample was wrapped in ashless filter paper, soaked in a solution of cellulose acetate in acetone and ethyl acetate and dried in an oven. This treatment bound the feathers in a compact mass and prevented scattering of the partly burned pieces during combustion.

An attempt was made to use the tetrahydroxyquinone titration method described by Brunjes and Manning ('40), for the determination of sulfate after the combustion of feed, excreta, or eggs. The high phosphate content of these materials, however, interfered with the determination of sulfate. Consequently, the A.O.A.C. gravimetric procedure was followed ('40). The tetrahydroxyquinone titration method was used for feathers because they did not contain enough phosphate to interfere.

When the hens had become accustomed to the confinement and the ration, a test period of approximately 3 months was begun. During this time the hens ceased laying, passed through a period of molting and then resumed egg production. Nine collections of 4 or 12 days' duration were made for each hen. These included periods of low egg production, molting and high egg production.

RESULTS AND DISCUSSION

As an example of the type of data obtained, the results of four balance periods with hen 1 are presented in detail in table 1. The four periods are representative of the effect on the sulfur balance of feather loss during molting, of no egg production, and of low and high egg production. The data of the thirty-five balance periods are summarized in figure 1.

TABLE 1

Average daily sulfur balances of representative periods.

	HEN 1			
	Period 3 (4 days)	Period 5 (4 days)	Period 11 (12 days)	Period 13 (12 days)
Body weight, gm.	1500	1500	1780	1870
Feed consumed, gm.	47	86	93	72
Sulfur in feed, %	0.167	0.169	0.170	0.169
Sulfur intake, mg.	78	145	158	122
Weight of air-dried droppings, gm.	18.8	25.5	27.0	22.0
Sulfur in droppings, %	0.343	0.324	0.357	0.416
Sulfur in droppings, mg.	64.0	82.0	96.5	92.0
Egg production rate, %	0	0	16	58
Dry weight of eggs, gm.	..	..	2.6	9.9
Sulfur in eggs, %	..	..	0.444	0.481
Sulfur in eggs, mg.	..	..	11.5	48.0
Weight of feathers, gm.	1.2 (molting)	0	0	0
Sulfur in feathers, %	2.09	..	..	..
Sulfur in feathers, mg.	25	..	..	..
Sulfur output, mg.	89	82	108	140
Daily sulfur balance, mg.	− 11	+ 63	+ 50	− 18

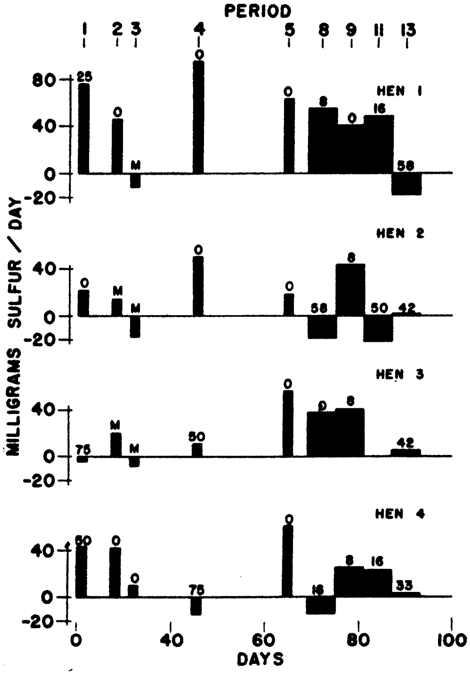

Fig. 1 Sulfur balances of hens. Numbers above the bars indicate the percentage of egg production. M indicates periods of molting in which the feather loss exceeded 0.5 mg. per day. Periods 1–5 were of 4 days' duration, periods 8–13 of 12 days' duration.

Period 1 (fig. 1), October 14th–18th, represents the close of egg production, periods 2 and 3 occurred during molting, and periods 4, 5, 8, 9, 11 and 13 represent a resumption and gradual increase of egg production. The study was terminated on March 8th. During molting the degree of sulfur retention was determined by the extent of feather loss. In some cases the sulfur balance was markedly negative and of the same order of magnitude as that which occurred during periods of high egg production. Thus for hen 2, period 3, the average daily balance was — 18 mg., which is of the same order as that observed later for the same hen when egg production was 58% and 50%. (Maximum egg production potentiality is considered to be one egg per day. The number of eggs produced divided by the number of days in the collection period times 100 is the percentage egg production.) The sulfur balances for these production levels were — 22 mg. and — 18 mg., respectively.

TABLE 2

Average daily sulfur balance and average daily sulfur excretion at various levels of egg production.

EGG PRODUCTION [1]	AVERAGE DAILY S BALANCE	AVERAGE DAILY S EXCRETION	NUMBER OF OBSERVATIONS
%	mg.	mg.	
0	+ 44.0	97	12
0 (molt)	— 0.6	81	5
8	+ 40.8	98	4
16	+ 19.7	121	3
25	+ 76.0	83	1
33	+ 0.2	100	1
42	+ 3.5	73	2
50	+ 10.6	93	3
58	— 18.0	93	2
75	— 11.0	98	2

[1] Maximum egg production potentiality is considered to be one egg per day. The number of eggs produced divided by the number of days in the collection period times 100 is the percentage egg production.

Although there was no close direct relationship between egg production and the daily sulfur balance (table 2), the average daily sulfur balance was found to be + 20 mg. or higher for levels of egg production of 25% and less. When production was greater than 25%, the balance was of the order of + 10 mg. or less, becoming negative for production rates higher than 50%. To maintain a hen in positive sulfur balance at high levels of egg production, the ration would have to contain a higher percentage of sulfur-containing substances or the feed consumption would have to be increased.

Even when sulfur demands are large, as in high egg production, the sulfur in the droppings, expressed as a percentage of that consumed, remains high. Thus in seven periods in which egg production was 50% or higher, the average value was 64%, in 13 periods of nonproduction 71%, and in 5 periods of molting 80%. That sulfur compounds are not retained to maintain sulfur equilibrium may be due to metabolic processes, such as methylation by methionine, in which the excretion of the demethylated portion of the methionine molecule, or degradation products from this portion, occurs. Another possibility is that the body has absorbed from the feed sulfur-containing compounds which are of no value in metabolic processes, egg production, or feather formation and are therefore excreted. A third possibility is that certain sulfur compounds are not absorbed and become a part of the fecal portion of the droppings.

SUMMARY

1. The sulfur balance of the hen has been determined during low and high egg production and during molting.
2. With an increase in egg production the sulfur balance decreased, becoming negative in most cases when the production rate went above 50%.
3. During the feather loss of the molting period, the sulfur balance decreased and became negative when feather loss was heavy.
4. Sulfur in the droppings, calculated as a percentage of that in the feed, remained high even during severe feather loss or high egg production.

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AVAILABILITY OF VITAMINS IN FOODS AND FOOD PRODUCTS

II. RIBOFLAVIN BALANCES IN DRIED LIVER, IN A LIVER VITAMIN CONCENTRATE, AND IN BREWERS' AND BAKERS' YEAST[1]

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(Received for publication December 27, 1944)

In the first paper of this series (Sure, '44a) it was shown that thiamine in three brands of brewers' yeasts of various potencies is well utilized but there was 6 to 11% poorer absorption of thiamine in one type of yeast compared with an equivalent intake of pure vitamin B_1.

In this communication results of riboflavin balance studies are reported on dehydrated liver, on dehydrated vitamin concentrate prepared from liver,[2] on three brands of dried brewers' yeasts, and on one brand of dried bakers' yeast.

EXPERIMENTAL

In order to further reduce the possibilities of bacterial synthesis in the fecal excretions (Sure and Ford, '42), the feces were collected twice daily, at 8 A. M. and at 4:30 P. M., then covered with petroleum ether in amber colored bottles and were kept in an electric refrigerator at about 30°F. for a week until they were ready for analysis. For the riboflavin content of feces we used the procedures of Conner and Straub ('41) and for the riboflavin content of urine we employed our recent modifications (Sure, '44b) of the method of Hodson and Norris ('39) for determining the riboflavin content of foodstuffs.

The riboflavin content of the dried liver was 60 μg./gm. and of the liver vitamin concentrate, 225 μg./gm. Of the dried liver, 61% of the riboflavin was found to be in the free form. Of the liver vitamin concentrate, 72% was found to be in the free form. The riboflavin content of brewers' yeasts A, B, C and the bakers' yeast were 50, 70, 40, and

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[1] Research paper no. 798 Journal Series, University of Arkansas. Published with the approval of the Director of the Arkansas Agricultural Experiment Station.

[2] Kindly furnished by Dr. David Klein of the Wilson Laboratories, Chicago, Ill.

46 μg./gm., respectively. Approximately 50% of the riboflavin in the yeasts was in the free form.

The animals were fed a purified synthetic diet of the following percentage composition: vitamin-free casein, 18; cellu-flour, 2; salts no. 1 (Sure, '41), 4; butter fat, 5; and cerelose, 71. The vitamin supplements were given daily separately from the ration, as follows: 20 μg. thiamine, 20 μg. pyridoxine, 6 mg. choline chloride, and 200 μg. calcium pantothenate. As a source of vitamins A and D, three drops of halibut liver oil were given once weekly to each animal. The daily doses of dried liver products and dried yeasts were administered in petri dishes.

For the riboflavin metabolism studies two groups of male rats, twenty-four in each set, were used. The animals of the first group were started on experiments when 54 days of age, weighing 85 to 124 gm. each. The animals of the second set were placed on experiments when 31 days old, weighing 72 to 88 gm. each. The first set was depleted of riboflavin for 64 days and the second set for 34 days. The period of depletion was determined by the characteristic signs of riboflavin deficiency, such as keratitis, cataracts, alopecia, and muscular incoordination. During the depletion periods two animals of the first set and one of the second set died. It was found in this investigation that the same animals could be used for balance studies of several foods. After the animals were well depleted of riboflavin, data on urinary and fecal excretions of this vitamin were secured, which were subtracted from results obtained following the administration of dehydrated food-products. The animals were then ready for riboflavin balance studies.

The results of this investigation are submitted in summarized form in table 1.

Liver

Riboflavin balance studies were carried out on twenty-two animals for 21 days, allowing 20 μg. of this vitamin daily. A daily dose of 333 mg. of the dried liver, which furnished 20 μg. riboflavin, was administered to ten animals. At the same time an equivalent amount of pure crystalline riboflavin was given daily to twelve other animals. The next set of rats received 40 μg. riboflavin daily, which brought the dose of dried liver to 666 mg. Twelve rats received 40 μg. of pure riboflavin daily and 11 animals were given 666 mg. dried liver daily which furnished the same amount of this vitamin. The latter metabolism experiments were continued for 14 days. It will be noted that the fecal riboflavin excretions of the animals on the dried liver were two and one-half to three times as great and the urinary riboflavin excretions

TABLE 1

Riboflavin balances in dried liver, in a liver vitamin concentrate, and in brewers' and bakers' yeasts.[1]

FOOD PRODUCT OR PURE RIBOFLAVIN	NO. OF ANIMALS	METABOLISM PERIOD	DAILY RIBOFLAVIN INTAKE	TOTAL RIBOFLAVIN INTAKE	CHANGE IN BODY WEIGHT	RIBOFLAVIN EXCRETED IN URINE [2]		RIBOFLAVIN EXCRETED IN FECES [2]	
		days	*µg.*	*µg.*	*gm.*	*µg.*	*% of T. I.*[3]	*µg.*	*% of T. I.*[3]
Dried liver	10	21	20	420	+ 76.6	44.9	10.7	177.4	42.2
Pure riboflavin	12	21	20	420	+ 57.2	24.8	5.9	70.4	16.8
Dried liver	11	14	40	560	+ 65.2	72.2	12.8	221.1	39.4
Pure riboflavin	12	14	40	560	+ 50.5	28.1	5.2	67.7	12.1
Dried liver vitamin concentrate	12	14	20	280	+ 11.9	44.4	15.9	173.6	62.0
Pure riboflavin	10	14	20	280	+ 15.7	33.7	12.3	64.8	23.1
Dried liver vitamin concentrate	12	14	40	560	+ 13.4	43.1	7.7	81.6	14.4
Pure riboflavin	11	14	40	560	+ 15.9	78.7	14.1	269.0	48.1
Brewers' yeast A	6	21	20	420	+ 56.0	39.0	9.0	127.0	30.2
Brewers' yeast B	6	21	20	420	+ 57.0	38.5	9.0	91.4	21.8
Brewers' yeast C	6	21	20	420	+ 48.5	54.8	13.0	178.4	42.5
Bakers' yeast	6	21	20	420	+ 53.0	57.8	13.8	170.9	40.7
Pure riboflavin	10	21	20	420	+ 40.0	47.1	11.2	107.1	25.5

[1] Figures in this table represent averages per animal.

[2] Corrected for the amount excreted on a riboflavin deficient ration.

[3] T. I. = Total intake.

two to two and one-half as great as the excretions of the animals which received the same daily dose of pure vitamin.

Yeasts

All the yeasts were fed daily in amounts which provided 20 µg. riboflavin, as follows: brewers' yeasts: A, 400 mg.; B, 286 mg.; C, 500 mg.; and bakers' yeast, 435 mg. The metabolism period was 21 days. From table 1 it is apparent that the animals on the various types of yeasts varied considerably in the fecal excretions of riboflavin, the significance of which will be discussed later in the paper. There was little change, however, in the urinary excretions of riboflavin during the corresponding periods on the various yeasts.

Liver vitamin concentrate

The dried vitamin concentrate prepared from liver was fed in daily amounts of 89 and 178 mg., which provided 20 and 40 µg. riboflavin, respectively. Equivalent amounts of pure riboflavin were fed to about the same number of animals. It is evident from table 1 that the animals which received the riboflavin from the liver vitamin concentrate excreted about three times as much of this vitamin in the feces as the rats which received the same amounts of the pure vitamin. There were also greater excretions of riboflavin in the urine in the animals which ingested the liver vitamin concentrate than in the rats which received the equivalent amounts of pure vitamin but the differences were not as marked as in the fecal excretions.

DISCUSSION

Since this study was completed the results of the work of Mannering, Orsini and Elvehjem ('44) appeared which demonstrated that the nature of the carbohydrate markedly influences the fecal riboflavin excretions. The substitution of 40% sucrose by an equivalent amount of lactose in the diet almost quadrupled the weekly riboflavin output. The authors attribute the increased fecal riboflavin excretions to bacterial synthesis in the intestinal tract.

The question arises whether the large increased excretions of fecal riboflavin following the feeding of the dried liver and the dried liver vitamin concentrate are of dietary or of bacterial origin. If the large excretions of fecal riboflavin on the liver products are due to poor absorption, then it would follow that the riboflavin in these food products is present in a combined form which is largely unavailable to the animal organism. However, as stated earlier in the paper, it was found by analysis that the riboflavin in these liver products is present mainly in the free form; hence, poor absorption is not the main factor contributing to the large fecal riboflavin excretions; these high excretions are probably due to a large extent to bacterial synthesis.

With regard to the large fecal excretions following the administration of various yeasts, two explanations are possible, particularly, since yeasts vary greatly in composition, depending on strain and the culture medium on which they are grown. Either yeasts contain variable constituents which may influence bacterial synthesis in the gut, thus resulting in greater fecal riboflavin output, or the latter may be due to poor absorption (50% of the riboflavin in yeasts being in combined form) because of the changes being produced in the yeasts by the method of dehydration.

No noteworthy differences were found in urinary excretions of riboflavin in the animals which secured this vitamin from the yeasts as compared with those which received the equivalent amounts of the pure vitamin. However, the rats on the dried liver and on the higher intake of liver vitamin concentrate excreted larger amounts of riboflavin in the urine than did the animals given the same amounts of the pure vitamin. The reason for this is not apparent, since definite information on absorption is lacking because of the complication of bacterial synthesis.

The greater gains in body weight of the animals which received the liver products and yeasts compared with the rats which received the synthetic vitamin B complex mixture may be due to the presence in the natural food-products of unidentified growth-promoting essentials. p-Aminobenzoic acid and inositol were not included in the mixture of the B vitamins, because they were found non-essential for growth of the rat in our laboratory (Sure, '43). Biotin and folic acid were not available.

SUMMARY

Albino rats fed dried liver and a dried vitamin concentrate prepared from liver as sources of riboflavin excreted much greater proportions of the total intake of this vitamin in the feces and urine than animals which were given equivalent amounts of this vitamin. Since the greater part of the riboflavin in these liver products exists in the free state, the large excretions of this vitamin in the feces could not be due to poor absorption; in all probability they were due to bacterial synthesis. In the yeasts about 50% of the riboflavin was present in the free form; therefore, the large fecal excretions of riboflavin of the animals which received the yeasts may have been of dietary or of bacterial origin.

The animals on the dried liver and on the higher intake of liver vitamin concentrate excreted larger amounts of riboflavin in the urine than the animals given the same amounts of the pure vitamin. The reason for this is not at all clear, since we have no definite information on absorption because of the complication of bacterial synthesis.

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JUL 1945

THE EXISTENCE OF A MICROBIOLOGICALLY INACTIVE "FOLIC ACID"-LIKE MATERIAL POSSESSING VITAMIN ACTIVITY IN THE RAT

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ONE FIGURE

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Milk has been found to contain only traces of "folic acid" (FA)[1] when assayed microbiologically following a standard digestion procedure (Cheldelin and Williams, '42). Welch and Wright ('44) have reported in a preliminary note that, although powdered whole milk does not contain significantly more FA, as determined microbiologically, than do the usual highly purified diets, the incorporation of succinylsulfathiazole (SST), in amounts up to 10% of a ration of powdered whole milk did not lead to a diet capable of producing a FA deficiency in the rat. These data were taken as evidence for the existence in powdered whole milk, and probably in other natural materials, of a component which may be utilized by the rat in lieu of material possessing the microbiological activity of FA. Since our note was submitted for publication Binkley et al. ('44) have offered evidence for the existence of a conjugate of FA (or vitamin B_c) in yeast extract that is available to the chick but not to Lactobacillus casei. FA could be released from the complex by "enzymatic digestion." Mims, Totter and Day ('44) have shown that after digestion with rat liver, according to the technique described by Wright and Welch ('43), large amounts of microbiologically active FA could be demonstrated in yeast extract and in certain other materials. Mallory, Mims, Totter and Day ('44) recently have shown that the growth-promoting and leucocytopoietic effects of yeast and liver extract were proportional to the total amount of FA found after enzymatic digestion rather than to their content of FA obtained by direct microbiological assay.

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[1] In this case the term "folic acid" includes those factors, probably closely related, which promote the growth of Lactobacillus casei and Streptococcus fecalis R.

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This paper presents additional evidence for the existence of a microbiologically inactive FA-like material in milk, which possesses vitamin activity in the rat.

PROCEDURE

Weanling rats of both sexes and from two different commercial sources were used in the growth studies with powdered milk. They were caged over wide-mesh screening and fed ad libitum. The 'Klim' brand of powdered milk was used throughout. The compositions of the rations of powdered milk used in the growth studies and of the purified diets that were fed to reference animals are given in table 1.

TABLE 1

Composition of diets.

COMPONENT	DIET NUMBER					
	S-9	S-10	S-23	S-30	S-31	M-1
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	
Milk powder, 'Klim'	...	...	...	...	...	1000
Vitamin-free casein	180	180	180	180	180	....
Sucrose	618	598	598	660	610	....
'Cellu' flour	40	40	40	...	...	....
Succinylsulfathiazole	...	20	20	...	50	....

Diets M-2, M-3, M-4, M-5, and M-6 prepared by mixing 990, 980, 950, 900, and 800 gm. of M-1 with 10, 20, 50, 100, and 200 gm. respectively, of SST.

In addition to the constituents given the purified diets (S-9 through S-31) contained (per kilogram): 100 gm. Primex; 20 gm. corn oil; 40 gm. salts (Hubbell, Mendel, and Wakeman ('37)); 10 mg. 2-methyl-1, 4-naptho-hydroquinone diacetate. Diets S-23, S-30, and S-31, and M-1 contained (per kilogram): 8 mg. thiamine chloride; 16 mg. riboflavin; 40 mg. nicotinic acid; 44 mg. calcium pantothenate; and 8 mg. pyridoxine, hydrochloride. Diets S-9 and S-10 contained in addition to the above B vitamins 80 mg. inositol and 40 mg. p-aminobenzoic acid per kilogram. All the diets contained (per kilogram); 1 gm. choline chloride and 1 gm. of vitamin A, D and E concentrate compounded as follows: fish liver concentrate containing 450,000 U.S.P. units of vitamin A and 90,000 U.S.P. units of vitamin D per gm. 7 gm.; α-tocopherol 2 gm.; corn oil 41 gm.

The values which are reported for FA are expressed in terms of the presumably pure L. casei factor [2] and were determined through the use of Lactobacillus casei according to the procedure of Landy and Dicken ('42). In addition, occasional comparative assays were carried out simultaneously with Streptococcus fecalis R according to the method of Mitchell and Snell ('41).

FA was determined microbiologically in the liver samples after a preliminary enzymatic digestion. Liver samples weighing 2 to 5 gm.

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[2] L. casei factor was kindly furnished by the Lederle Laboratories.

were fragmented in 20 ml. of water. An amount of takadiastase equivalent to 2% of the weight of the liver was added and digestions were carried out for 18–24 hours at 37°C. under benzene. This, of several digestion procedures, has resulted in the highest values for FA in rat liver (Wright, Skeggs and Welch, '45).

The FA content of the feces was determined on complete 24-hour samples. Blotting paper was placed beneath the cages of three animals in each group and fecal material was transferred to the refrigerator at frequent intervals throughout the day. The combined 24-hour collection of feces from each group of rats was weighed and digested in 10 volumes of 1% acetate buffer (pH 4.0) with an amount of takadiastase equivalent to 2% of the weight of the sample. Digestions were carried out under benzene for 18–24 hours at 37°C. As an inhibitor of sulfonamides 10 mg. of para-aminobenzoic acid was added to each 100 ml. of medium used for microbiological assay.

Powered milk and the purified diets were assayed for FA following digestion according to the procedure for foods described by Cheldelin et al. ('42).

RESULTS

The values tabulated below summarize the results of the microbiological determinations for the FA content of the powdered milk and highly purified diets employed: L. casei assay — milk powder rations, 0.016 μg./gm., highly purified rations, 0.010 μg./gm.; and S. fecalis R assay — milk powder rations, 0.021 μg./gm., highly purified rations, 0.00 μg./gm.

Various modifications of the Cheldelin procedure, such as the use of increased amounts of takadiastase, digestion in water, or the direct extraction by autoclaving with water or buffer have failed to increase the values for FA obtained by microbiological assay. The occurrence of only small amounts of FA in these diets required their assay at unusually high levels. In addition, certain naturally occurring purines and pyrimidines have been found to stimulate the growth of lactic acid organisms in the absence of FA. These facts tend to indicate that the values reported are probably considerably higher than the actual amount of FA present.

In figure 1 are presented the data on the growth of the animals fed the various milk diets. Accompanying the curves are the proportionate number of male and of female rats involved in the various groups. The values plotted are the averages obtained from three separate experiments. They do not include the weight changes in the animals that failed to survive the test period or in representative animals killed for analysis. There were no deaths recorded that could be attributed to the inclusion of less than 10% SST in the diets of powdered milk. Poor growth, and the death of four of eight rats within a period of 8 weeks, resulted when a diet of powdered milk containing 20% SST was fed. At the 10% level growth was depressed but little and only one of eleven rats failed to survive a feeding period of 14 weeks duration. Growth was entirely normal when SST was added to the diet of powdered milk in amounts of 5, 2 or 1%.

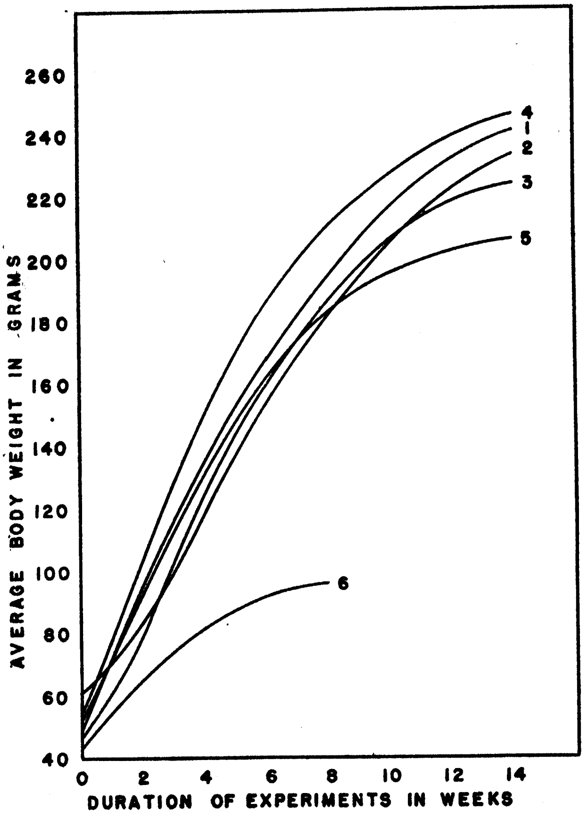

Fig. 1 The growth curves of rats ingesting various milk powder diets. (1) 0% SST, 7M, 4F; (2) 1% SST, 2M, 3F; (3) 2% SST, 2M, 3F; (4) 5% SST, 7M, 7F; (5) 10% SST, 3M, 8F; (6) 20% SST, 3M, 1F.

A previous study has shown that a deficiency of FA in the rat is accompanied by a low FA content in the liver (Wright and Welch, '44). After the rats had received the diet of milk powder for 5 weeks, and at intervals thereafter, determinations of FA were made on the livers of several rats of each group. No variation was found to occur in the hepatic level of FA after the fifth week. The values given in table 2 represent group averages obtained over the remaining period of feeding. Included also are reference data for storage of FA in the livers of stock animals, of rats receiving adequate highly purified diets, and of rats receiving highly purified diets containing sufficient SST to induce a deficiency of FA. The hepatic levels of FA were obtained in the latter group when the animals had reached a plateau in growth.

TABLE 2

The "folic acid" contents of the livers of rats receiving various diets.

TYPE OF DIET	LEVEL OF SUCCINYL-SULFATHIAZOLE	CONDITION OF ANIMALS	"FOLIC ACID"[1] CONTENT		
	%		μg./gm.		
Stock	0	good	9.7	(8.4–12.0)	(3)
Purified (S-9)	0	good	1.7	(0.79–4.0)	(4)
Purified (S-10, S-23)	2	deficient	0.40	(0.13–0.73)	(10)
Purified (S-31)	5	deficient	0.30	(0.20–0.35)	(6)
Milk powder (M-1)	0	good	3.3	(1.6–6.4)	(8)
Milk powder (M-2)	1	good	2.9	(1.2–5.3)	(4)
Milk powder (M-3)	2	good	1.8	(1.3–2.9)	(4)
Milk powder (M-4)	5	good	0.95	(0.45–1.6)	(10)
Milk powder (M-5)	10	good (?)	0.45	(0.30–0.68)	(10)
Milk powder (M-6)	20	deficient	0.28	(0.17–0.44)	(6)

[1] The results given are in terms of the fresh weight of the liver employed. Accompanying the average values presented are (1) the range in values obtained, and (2) the number of liver samples studied.

A level of approximately 0.5 μg. (or less) of FA per gram of liver has been found to be associated with a cessation of growth and with other evidences of a deficiency of FA. The inclusion of 2% SST in highly purified diets readily induced such low hepatic levels. In contrast, it was necessary to incorporate from 10 to 20% SST into a powdered milk diet in order to obtain such a correspondingly low level of FA in the liver.

The effect on the fecal elimination of FA of feeding a diet of powdered milk, as compared with other diets, is shown in table 3. The inclusion of large amounts of SST in the milk diets decreased the amount of FA eliminated daily in the feces. However, the incorporation of as much as 20% SST in the powdered milk diets did not decrease the fecal elimination to a value comparable to that produced by the inclusion of 2% SST in a highly purified diet. It appears from the data in table 3 that, while there is a relationship between the elimination of FA in the feces and the occurrence of signs of deficiency, there is no critical level of FA elimination below which deficiency signs definitely will occur.

TABLE 3

The "folic acid" contents of feces of rats receiving various diets.

TYPE OF DIET	LEVEL OF SUCCINYL-SULFATHIAZOLE	CONDITION OF ANIMALS	"FOLIC ACID"[1]		
	%			*µg./rat/day*	
Stock	0	good	49	(34–74)	(6)
Purified (S-30)	0	good	2.1	(1.5–2.6)	(2)
Purified (S-23)	2	deficient	0.51	(0.43–0.60)	(3)
Milk powder (M-1)	0	good	6.8	(2.2–20.8)	(7)
Milk powder (M-4)	5	good	1.4	(0.79–2.6)	(5)
Milk powder (M-5)	10	good (?)	2.0	(1.0–3.6)	(5)
Milk powder (M-6)	20	deficient	3.9	(1.4–6.5)	(2)

[1] Accompanying the average values presented are (1) the range in values obtained, and (2) the number of assays performed.

Representative animals from the control and from each sulfonamide-fed group were autopsied at the conclusion of the experiment. The histological changes encountered were for the most part limited to lesions in the kidneys, ureters and bladder produced by the deposition of concretions of sulfonamide. Concretions were encountered in two of three animals examined in the 5% group (14 weeks of feeding), in all of three animals in the 10% group (14 weeks) and in five of six animals in the 20% group (8 weeks). In only one animal of six in the 20% SST group was there encountered hyperplasia of the thyroid, as described by Daft et al. ('42), Mackenzie and Mackenzie ('43) and Astwood et al. ('43). Mild involutional changes of the thyroid were encountered in the three animals examined in the 10% group. In none of the animals were there observed any lesions of the myocardium, vascular system or liver such as were described by Daft et al. ('42).

At the conclusion of the experiments in which dried milk and SST were fed, the total and differential leucocyte counts of representative animals were determined. The results are summarized in table 4. Accompanying these data are the results of similar determinations performed with normal stock animals. For brevity, only the percentage of neutrophils in the differential count is given. Rats which had been fed 20% SST in rations of powdered milk were found to have a definitely lowered total leucocyte count and a decreased percentage of circulating neutrophils. In agreement with the previous data on growth and on hepatic storage, the animals given 10% SST might be classed as "borderline" with reference to the possible existence of a deficiency of FA. These animals were found to have a somewhat lowered total count although the percentage of circulating neutrophils was normal. There was no evidence of blood dyscrasia in the animals ingesting less than 10% SST in diets of powdered milk for as long as 14 weeks. Occasional bacterial counts (Strawinski, Verwey and Munder, '45) were made on the feces of rats fed the powdered milk diets. The fecal flora, with respect to number and kind of bacteria, was similar to that of rats fed highly purified diets of comparable sulfonamide content.

TABLE 4

The total leucocyte counts and percentages of neutrophils in the blood of rats receiving various diets.

TYPE OF DIET	LEVEL OF SUCCINYLSULFATHIAZOLE	CONDITION OF ANIMALS	DURATION OF FEEDING	TOTAL COUNT [1]	NEUTROPHILS [1]
	%		*weeks*	*thousand/cmm.*	%
Stock	0	good	..	13.0 (8.9–17.2) (16)	23.6 (13.7–35.0) (16)
Milk powder	0	good	14	10.8 (6.5–16.4) (4)	....
Milk powder	1	good	14	11.5 (7.6–15.5) (2)	....
Milk powder	2	good	14	14.7 (8.5–21.0) (2)	....
Milk powder	5	good	14	12.8 (4.6–21.7) (5)	15.3 (10.5–23.0) (5)
Milk powder	10	good (?)	14	6.3 (3.3–9.0) (6)	19.2 (15.0–25.0) (6)
Milk powder	20	deficient	8	6.3 (3.2–8.9) (3)	9.6 (5.0–18.5) (4)

[1] Accompanying the average values presented are (1) the range in values obtained, and (2) the number of determinations performed.

DISCUSSION

Data summarized in the foregoing sections have supplied considerable evidence that diets of powdered milk, although extraordinarily low in FA as usually determined microbiologically, have a marked capacity to protect the rat against the development of a deficiency of FA, as is produced by small amounts of SST in highly purified diets.

It has been demonstrated that critically low hepatic levels of FA could be produced by adding large amounts of SST to diets of powdered milk. The fecal elimination of FA remained relatively high despite the large amount of SST in the diet. This would indicate either that the utilization of FA may have been impaired by the addition of the drug to the diet or that increased intestinal synthesis of FA was not a primary factor in protecting the rat against FA deficiency produced by the drug.

We have attempted to utilize various techniques in vitro to supplement the conclusions derived from animal feeding experiments. Studies on rat liver, incubating in vitro, have indicated the existence in milk of significant amounts of "potential FA"; that is, a substance which could be measured microbiologically after the action of liver enzymes (Wright and Welch, '43). Since the "observed FA" content of rat liver may be modified by many factors (Wright, Skeggs and Welch, '45) it is difficult to assign more than qualitative significance to the results of these incubation studies. The use of purified preparations of the enzyme in rat liver capable of "activating" the non-microbiologically active "potential FA" is not subject to these limitations (Mims, Totter and Day, '44). Through the kind cooperation of Dr. Totter samples of the powdered milk used in our diets have been assayed for FA following incubation with the partially purified enzyme. Dr. Totter has reported to us that samples of powdered milk contain 0.48 to 0.60 µg. of FA per gram after such digestion (while before digestion only 0.016 to 0.021 µg. could be found).

If a growing rat may be assumed to ingest 10 gm. of the diet of powdered milk per day, such consumption would supply at the most a dietary intake of only 0.2 µg. of microbiologically active FA. Although quantitative data for the dietary requirement of the rat for FA have not as yet appeared, the ease with which a deficiency of FA may be produced with purified diets containing comparable amounts of FA and only 0.5 to 2% of SST would indicate that the daily requirement of FA exceeds 0.2 µg. Also, it has been shown (Welch and Wright, '43) that 5 to 6 µg. of FA per day, in conjunction with biotin, will cure the signs of deficiency of FA induced by the ingestion of highly purified diets containing 2% SST. Since a rat consuming approximately 10 gm. of the diet of powdered milk per day ingests 5–6 µg. of "potential FA," it seems justifiable to conclude that bovine milk contains a naturally occurring form of FA which serves in lieu of or as a source of FA in the metabolism of the rat, but which is not utilizable as such by the lactic acid bacteria commonly employed in microbiological assays.

SUMMARY

1. In order to produce the syndrome of "folic acid" (FA) deficiency in the rat it is necessary to include much larger amounts

of succinylsulfathiazole (SST) in diets of powdered milk than in highly purified diets of comparable FA content.

2. The deficiency resulting from the feeding of diets composed of powdered milk and large amounts (10 or 20%) of SST is characterized by a failure in growth, the attainment of low hepatic levels of FA, a low total leucocyte count, and a low percentage of circulating granulocytes.

3. Large amounts of FA are eliminated in the feces of rats fed exclusively on powdered milk. This fecal elimination of FA is reduced by feeding SST, but a syndrome of FA deficiency can exist in the presence of a considerable fecal elimination of the vitamin.

4. By the use of liver digestion techniques or by the use of a more highly purified enzyme capable of releasing FA from a non-microbiologically active complex, it was possible to show that powdered milk contains significant amounts of "potential FA."

5. It is concluded that the "potential FA" of milk, unavailable as such to microorganisms, is utilizable by the rat. The existence of such "potential FA" offers an explanation for the apparent discrepancy between the FA content of milk powder found microbiologically and that indicated by the results of growth experiments in rats.

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MITOTIC ACTIVITY AND WOUND HEALING IN THE CORNEAL EPITHELIUM OF VITAMIN A DEFICIENT RATS[1]

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ONE FIGURE

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The present study is one of a series directed toward discovering factors which control the mitotic and wound healing activity of the corneal epithelium (Buschke, Friedenwald and Fleischmann, '43; Friedenwald and Buschke, '44a, '44b). Since vitamin A deficiency gravely affects this tissue, we were interested to find out whether and in what way the activities which we were studying were influenced by such deficiency. Presumably because of the general retardation of body growth, Rosenberg ('42), has inferred that "Vitamin A can be regarded as a stimulus to the building of new cells." On the other hand, Wolbach and Bessey ('42) and Wolbach and Howe ('25, '33) state that in vitamin A deficient tissues "the rate of growth is rapid as attested by numerous mitoses of the basal cells," and they conclude that the growth activity of the epithelium is greatly augmented in vitamin A deficiency.

In view of this contradictory state of affairs, it would be desirable to obtain quantitative data on the "growth activity" of tissues in vitamin A deficiency. Attempts in this direction have heretofore been made mainly in regard to the healing time of larger wounds (Arey, '36 rev.; Buschke, '36 rev.; Heinsius, '37; de Roetth, '40). The methods employed in those studies, however, do not permit any differentiation between mitotic activity, non-mitotic cell movements, and — in at least some of the earlier studies — possible beneficial effects of vitamin A on secondary infections. It is perhaps due to this complexity of experimental factors, that the results obtained in various laboratories were not in agreement. In the present paper we have used methods of study which

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make it possible to investigate the effects of the deficiency on mitotic activity and on cell movements in wound healing separately.

In view of the normal variation of mitotic activity from animal to animal, the experiments were performed on a sufficiently large group to make possible valid statistical analyses of the results. Seventy-four rats were used in this study to estimate mitotic activity and post-traumatic cell movements, thirty-six vitamin A deficient and thirty-eight control rats. In addition, about 1 dozen animals kept on the A-deficient diets, developed severe corneal lesions typical of the vitamin A deficiency and were no longer amenable to a quantitative interpretation on the two cell functions under investigation. These latter animals served as qualitative guides and controls for the time course in our feeding experiments.

METHODS

The feeding experiment. Five groups of 3–4 weeks old albino and hooded rats of between 25 and 50 gm. weight were put on the experimental diets. The feeding time extended over a period of from 5 to 9 weeks, and the experiment was conducted during the months of January to July.

The diet used in four of these five groups was identical with that described by Sullivan and Evans ('42) in their "experiment I".[2] In this diet the vitamin A supplement for the control animals is supplied in fish liver oil which contains vitamin D in addition to vitamin A. One experiment was carried out without this extra vitamin D through supplying the vitamin A supplement for the controls in the form of carotene in oil.[3] Since the results in this group were essentially the same as those in the four other groups, our final statistics include all five groups without differentiation. The animals were kept in individual feeding cages with raised wire net bottoms. The rats were weighed once a week. The food intake of the deficient rats did not differ significantly from that in the control groups except in the latest stages of the deficiency.

The histological methods. The eyes were enucleated under ether anaesthesia, care being taken that the animals had not been excited for at least 2 hours preceding enucleation (Friedenwald and Buschke, '44b). The eyes were fixed in formalin-alcohol, and flat preparations of the corneas were made as previously described (Buschke, Friedenwald and Fleischmann, '43).

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[2] The alpha-tocopherol used as vitamin E supplement was kindly supplied by Merck and Co., Rahway, N. J.

[3] SMA CO.

The methods of assay. The methods of assay of the number and rate of mitoses have been previously described (Buschke et al., '43). The mitotic rate was determined by mitosis counts in rats who had been injected with 5 mg. colchicine per kilogram body weight 4 hours prior to enucleation. These counts as well as those with colchicine were made on flat preparations of the corneas.

The method of assay for post-traumatic cell movements in the rat's corneal epithelium has been described elsewhere (Friedenwald and Buschke, '44a). In the present experiments, the eyes were removed for histological study 3 hours following the injury.

RESULTS

The animals were sacrificed and enucleations were made when the deficient rats had stopped gaining or began losing weight. This varied from 5 to 9 weeks after institution of the experimental diets. At this time some of the animals in each series showed some other manifestations of the deficiency: Bloody exudate from the nose, blood stain of the tails, alopecia of the lids, extensive depigmentation of the incisor teeth, conjunctivitis, xerosis of the conjunctiva, and in some cases, beginning epithelial irregularities and slight opacification of the cornea. Animals which showed more severe changes of the corneas were not included in the quantitative study. Much to our surprise, the results on mitotic activity which will be statistically evaluated below, did not bear out any quantitative parallelism between the presence and severity of the symptoms enumerated here and the mitotic activity in individual cases. Therefore, no useful purpose would be served here by giving detailed descriptions or individual "case histories" of the progress of the deficiency. There was, however, a statistically significant difference between the mitotic activity in the deficient animals on the one hand, and that of the control animals on the other hand.

I. Mitosis counts

Mitosis counts in a meridional strip 0.11 mm. in width and crossing the whole cornea ranged from 2 to 142 with an average of 70, in twenty eyes of the deficient animals. In the control rats mitosis counts on twenty-seven eyes ranged from 75 to 206 with an average of 106. The critical ratio [4] of the means of these two groups is 3.2, and the difference therefore can be considered as statistically significant.

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[4] Critical ratio $= \dfrac{m_1 - m_2}{\sqrt{\sigma_{m_1}^2 + \sigma_{m_2}^2}}$

The interpretation of this finding is, however, not directly apparent. In connection with the general arrest of growth the length of the corneal strip was somewhat less in the deficient rats than in the controls. In no case did the difference in corneal diameters exceed 20% and the average difference was less than this. It may be concluded, therefore, that the number of mitoses per square millimeter of tissue was significantly less in the deficient than in the control animals. If, however, it is desired to compute the mitosis rates not in terms of tissue area but in proportion to the total number of cells present a further correction has to be made. As will be pointed out below the epithelial cells in the corneas of the deficient rats are often larger than those in the controls. In extreme cases the cells may be as much as twice as large in the deficient, consequently the number of cells is on the average less in the deficient than in the controls. Counts of the number of cells in the basal layer of the corneal epithelium were made on six deficient and six control eyes. These counts were made on the same meridional strips 0.11 mm. in width which had previously been used for mitosis counts. The specimens selected for the basal cell counts were chosen from those that showed the high, low, and medium numbers in the mitosis counts and were therefore representative of the average and of the range of variation in mitosis counts for the deficient and for the control group respectively. In the six corneas of deficient animals the mitosis counts ranged from 2 to 142 with an average of 71. The basal cell counts ranged from 5,676 to 12,024 with an average of 9,231. In the six corneas of control rats the mitosis counts ranged from 76 to 206 with an average of 116. The basal cell counts ranged from 10,968 to 13,194 with an average of 12,170. Thus there were approximately 25% fewer cells in the basal layer per strip in the deficient than in the controls. Consequently the number of mitosis per 1000 basal cells is not significantly reduced in the deficient animals, though the number of mitoses per square millimeter is significantly reduced.

II. Mitotic rate

As has been pointed out elsewhere, the absolute number of mitoses present in a certain area or per thousand basal cells, does not provide a complete answer as to the mitotic activity of the tissue. A relatively low number of mitoses may be found either with a decreased rate of onset (mitotic rate) or with an increased speed of the mitotic cycle. The mitotic rate can, however, be gauged with colchicine which leads to an accumulation in metaphase of all mitoses which have entered the mitotic cycle during a given period of time (Buschke, Friedenwald, and Fleischmann, '43).

Counts were made with our method 4 hours after an intramuscular injection of colchicine, on the corneal mitoses in thirty deficient and thirty-six control animals. In the deficient group the counts varied from 50 to 344 with an average of 155. In the control rats figures of between 148 and 890 were found with an average of 335. The critical ratio of the means of these two series of data is 7, and the difference, therefore, can be considered as certainly significant, in spite of the wide scattering of the extreme values. These figures refer to the mitosis rates per square millimeter. If correction is made for the fact that the number of basal cells is less in the deficient animals the mitosis rate per 1000 basal cells is found to be approximately 30% less in the deficient than in the controls. Allowing for the standard deviations in the basal cell counts this figure is still statistically significant.

Thus a decrease of the overall mitotic rate of about 30% is found in the vitamin A deficient animals, or — neglecting the duration of the mitotic cycle itself — a prolongation of the intermitotic rest period by approximately 40%.

III. Speed of mitotic cycle

The mitotic rate per 1000 basal cells as estimated from the colchicine experiments was reduced in the deficient rats by 30%, but the number of mitoses computed on the same basis found in the counts without colchicine was not significantly reduced. The significance of this can be seen as follows. If the duration of the mitotic cycle were normal, the mitosis counts without colchicine should show the same percent decrease as in the counts with colchicine. On the other hand, if the mitotic cycle were slowed down in the same proportion as is the rate of entrance into mitosis, then the mitosis counts without colchicine should show no difference in the deficient rats as compared with the controls. It is evident from this that the mitotic cycle is somewhat slowed down in vitamin A deficiency in approximately the same degree as is the rate of onset of mitosis.

IV. Individual mitotic phases

More detailed information about the speed of the individual phases of the mitotic cycle may be obtained by determining the phase distribution (Buschke, Friedenwald, and Fleischmann, '43).

The individual phases of mitosis (prophase, metaphase, anaphase, telophase, reconstruction phase) per hundred mitoses were determined in fourteen corneas of A-deficient rats and in seventeen corneas of control rats. No significant difference between these two groups was found in respect to the phase distribution. It can be concluded that the slowing of the mitotic cycle described in the previous section extends uniformly over the whole mitotic cycle and does not affect any phase predominantly, once mitosis has gotten under way. The effect is thus fundamentally different from the action of colchicine which arrests mitosis in metaphase.

V. Attempts at restitution from mitosis inhibition

For methodical reasons, it is difficult to test for the reversibility of the mitosis inhibition in vitamin A deficiency: As has been shown in section *I* and *II,* the inhibitory effect need not be manifest in any individual case, although statistically the phenomenon can be shown to exist. However, in one case where the number of mitoses in an ordinary count was down to 2 in one eye and where also some qualitative histological changes (see below, section *VI*) were found, vitamin A was supplemented; 9 days later the number of mitoses was counted in the second eye and found to be 110.

Similar experiments cannot be carried out in regard to the mitotic rate, as tested with colchicine, because the dose of this drug used in these experiments is fatal in the majority of animals after 12–24 hours.

We have attempted also to test the effect of supravital application of vitamin A on the mitotic rate following enucleation of the eyes of A-deficient colchicine injected animals. The results are not conclusive.

VI. Post-traumatic cell movements

Following superficial epithelial injuries, the cells of the corneal epithelium undergo characteristic changes in shape, orientation and position which may easily be seen on stained flat preparations (Friedenwald et al., '44a). These cell movements lead to the covering of small defects of about 30 microns diameter within a period of about 3 hours without the participation of mitosis.

Small epithelial injuries (needle pricks) were produced in the corneal epithelium of twelve eyes of A-deficient rats and in eleven eyes of control rats. Three hours later the eyes were enucleated and fixed for histological study. Only one of these twelve showed a significant inhibition of the epithelial cell movement. Several of the other eyes which failed to show any inhibition of the wound healing cell movements, did show some qualitative signs of A deficiency in the corneal epithelium. All of the control eyes showed normal post-traumatic cell movements.

VII. *Histologic observations*

In view of the excellent descriptions of the histopathology of A-deficiency in the cornea (Wolbach et al. '25, '33, '42; Goldblatt and Benischek, '27), it will be sufficient here to describe briefly merely one feature which is particularly apparent in stained flat preparations. A peculiar feature in some flat preparations of A-deficient corneas is the enlargement of the horizontal diameter of the basal layer of cells and of their nuclei to as much as twice their normal size (fig. 1). As noted above the deficient animals showed on the average 25% fewer cells in the basal layer of the corneal epithelium than did the normal. Between 10% and 15% of this decline is to be attributed to the fact that their corneal diameters are slightly smaller. The remaining discrepancy is a measure of the average increase in cell size. This measure, however, refers only to the relative sizes of the cells in their horizontal dimensions. In order to estimate the average cell volume it is necessary to know also the altitude of the cells. For this purpose sagittal histological

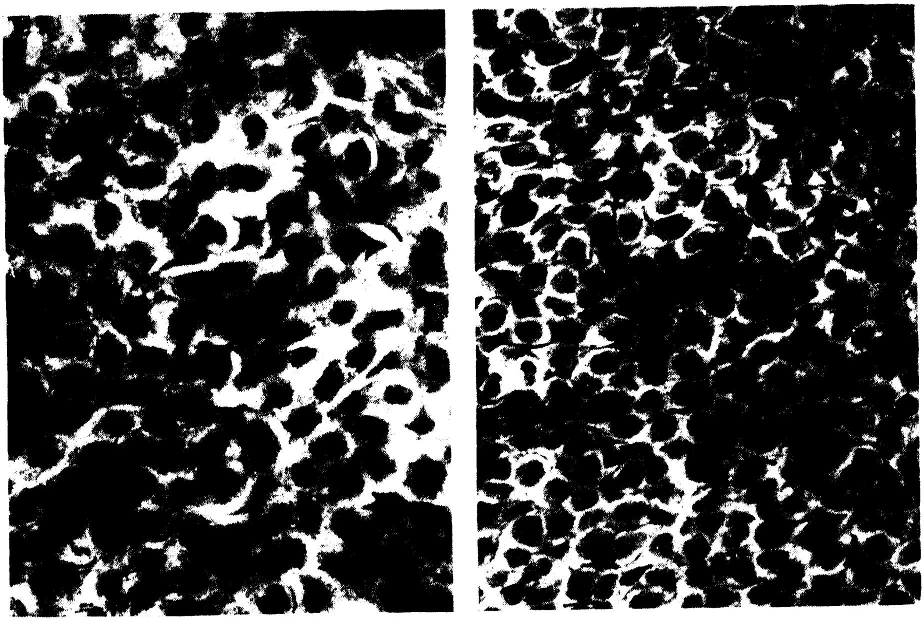

Figure 1

A

Corneal epithelium of A-deficient rat

B

Corneal epithelium of control rat

Flat Preparations Harris' Hematoxylin

sections were made of several eyes which showed an average or an extreme horizontal enlargement of the cells and these were compared with corresponding samples from among the controls. The vertical diameter of the cells of the basal layer of epithelium was found to be about 10% less in A-deficient rats than in the control rats; since the horizontal diameters were on an average about 15% larger in the A-deficient group, the cell volume of the basal epithelial layer is somewhat larger in this group as compared with the control group. The decreased rate of mitosis cannot, therefore, be attributed to a failure of the growth of the individual cells. Some of the large cells are markedly elongated.

DISCUSSION

Our experiments show that there is a profound, though variable inhibition of mitotic activity in the corneal epithelium of vitamin A deficient rats. Whether the variations in the inhibition represent cyclic variations in the individual rat or variations from rat to rat we cannot say. What is perhaps significant is that the severity of the inhibition was not found to be correlated with the severity of other grosser symptoms of vitamin A deficiency. As to the mechanism of the inhibition, no positive conclusions can be drawn at the present time. It may be pointed out, however, that among a large number of experimental inhibitors of mitosis which we have so far studied, only two others resemble vitamin A deficiency in inducing a simultaneous decline in the rate of entrance into mitosis, and in the rate of progress through the mitotic cycle. These two other inhibitors are barbiturates and the effect of removing the superior cervical sympathetic ganglion (Friedenwald et al. '44b). No suggestions can be made as to a common denominator among these three very dissimilar experimental conditions.

The recent literature contains a number of reports (Tansley, '36; Johnson '39, '43; Hale '33, '35) on the production of a variety of developmental anomalies in vitamin A deficient embryos and young animals. In view of our findings, it would seem possible that these anomalies may result from deficient mitotic activity in certain tissues.

In contrast to the marked inhibition of mitosis produced by vitamin A deficiency in the corneal epithelium, we have failed to find any marked reduction of those non-mitotic cell movements which are concerned in the healing of small wounds of the corneal epithelium. This contrast perhaps accounts for some of the disagreements among previous investigators in respect to the influence of vitamin A deficiency on growth and repair.

SUMMARY

1. The mitotic activity and the cell movements in wound healing have been examined in the corneal epithelium of vitamin A deficient rats.
2. The overall mitotic rate per 1000 basal cells was found to be reduced by about 30% in vitamin A deficiency.
3. The speed of the mitotic cycle was likewise found to be similarly reduced.
4. The epithelial cells in some vitamin A deficient animals were found to be considerably enlarged in their horizontal diameters. Consequently the inhibition of mitosis cannot be attributed to a failure in the growth of the individual cells.
5. The post-traumatic cell movements in the healing of small wounds in the corneal epithelium were not found to be significantly delayed.

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STUDIES ON THE COMPARATIVE NUTRITIVE VALUE OF FATS

VI. GROWTH AND REPRODUCTION OVER TEN GENERATIONS ON SHERMAN DIET B WHERE BUTTERFAT WAS REPLACED BY A MARGARINE FAT [1]

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TWO FIGURES

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In the recent paper of Boutwell, Geyer, Elvehjem, and Hart ('43), it was reported that growth was as satisfactory when various oleomargarines were incorporated in the diet as when butter was employed where the main carbohydrates were glucose, sucrose, dextrin, starch or a mixture of them. These authors state, however, that a final answer can only be obtained if similar results can be demonstrated on experiments in which growth to maturity and reproduction are studied. The present experiments which have been continued over 4½ years would seem to offer such proof.

Sherman and Campbell ('37) point out that diets which are nutritionally adequate may not, however, be optimum. An example of such an adequate diet is afforded by their diet A which had supported growth and reproduction for forty generations according to their last report, at which time the animals were still thriving. When the whole milk powder in this diet was increased from one-sixth (diet A) to one-third (diet B), a marked improvement in a number of biometric measurements which are generally regarded as indices of the nutritive value of a diet resulted (Sherman and Campbell, '24). Thus, the rats were larger at weaning, they grew at a faster rate and were larger at all ages, the duration during which reproduction was possible in the females was practically doubled, and an increased number of rats was reared. Also, a larger proportion of the rats were born and successfully raised through weaning on diet B than on diet A, the total failures being reduced from 48 to 19%. In a later study, it was also found that the span of life was prolonged by a statistically significant period in those rats which received diet B (Sherman and Campbell, '29–'30). These authors have concluded more recently (Sherman and Campbell, '37) that the superior nutritive value of diet B is to be ascribed to the increased content of calcium, vitamin A and riboflavin that it contains. The action of these factors is independent. Butterfat, for example, actually slows down the rate of growth, both of males and females, although its addition results in an extension of adult vitality and an increase in the life span with eventually a higher body weight being reached. However, the age at which females bore their first young was not decreased by the addition of butterfat to diet A.

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The present experiments were designed to determine whether the superiority of diet B is to be ascribed to the butterfat itself or merely to the fact that this component in the diet is the vehicle whereby an adequate quantity of vitamin A is assured. Earlier results from this laboratory (Deuel et al., '44 a, '45) have indicated that butterfat per se is not required for the growth of rats but that just as satisfactory results are obtained when it is replaced by corn, cottonseed, olive, peanut or soybean oil, or a margarine on a diet in which adequate amounts of the fat-soluble vitamins are supplied. It was also found that diets containing the above fats were equally satisfactory for pregnancy and lactation in the rat (Deuel et al., '44 b). However, these studies were made on only one generation; in the present study it was desired to determine whether a diet as Sherman outlines will prove adequate over a number of generations when the butterfat is replaced by a vegetable fat to which are added sufficient amounts of the fat-soluble vitamins. The fat chosen was from a commercial vegetable fat margarine purchased on the open market which is fortified with vitamin A.[2] Adequate quantities of vitamins D and E were present in the fat.

EXPERIMENTAL

The diet employed was similar to diet B used by Sherman and Campbell except that a margarine fat was employed in place of butterfat. Its composition is given in table 1.

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[2] The margarine used for the first two generations had a declared vitamin A potency of not less than 7500 I.U. per pound and that subsequently employed not less than 9000 I.U. per pound. On the basis of bioassay determinations made in our laboratory, we can state that the earlier samples of this margarine contained approximately 12,000 I. U. per pound and the subsequent ones used for the last eight generations contained approximately 15,000 I.U. per pound.

In addition to this diet, each rat received 5 gm. of lean meat and of lettuce once weekly after weaning. Campbell and Sherman ('38) have found that the feeding of fresh meat and green beans three times weekly to rats fed their diet B to some extent further improved the diet.

Twenty-one day weanling rats from our stock colony were started on the modified Sherman-Campbell diet in April, 1940. These are listed in the figures as generation 1. They were bred when they reached 3 months of age through the fifth generation after which they were mated when 4 months old. The group listed as first litter rats were the descendents of the original group through the first litters. The average age of the females when their litters were born was between approximately 110 and 120 days through the fifth generation, and 150 days in the later generations. The series marked "second litter rats" were continued through the second litter of the second litters of each generation. The rats were bred at the same age as the first litter series and then rebred 1 week after the weaning of their first litters. In all cases the young rats were weaned at 21 days. In general, from each litter one male and two female rats which weighed the most at weaning were used for the growth experiments. The average age of the females when the second litters were born was between 171 and 190 days for the second to fifth generation and from 200 to 215 days in the generations following.

TABLE 1

Diet 57 — Modified Sherman Diet.

INGREDIENTS		COMMENT
	%	
Skimmed milk power	23.76	Challenge brand. Contained approximately 1.10% of lipid.
Margarine fat	9.24	This proportion of added margarine fat gives the fat content equivalent to that which would be present if 33% of whole milk powder (containing 28% fat) were used.
Ground whole wheat	66.00	The wheat was ground as needed, using a Hobart mill.
Sodium chloride	1.0	

The experiments on stock rats were carried out during the winter of 1943–44 simultaneously with the ninth and tenth generations of the first litter rats, which received diet 57. All rats were originally from the same strain.

RESULTS

Figures 1 and 2 show the average weights of the various groups of male and female rats, respectively, at 21, 30, 60 and, in some cases, 90 and 120 days of age, as well as comparative values for stock rats maintained on our stock diet (diet 2)[3] as well as the values obtained by Sherman and Campbell ('24) and the standard values of Donaldson ('24). The averages are in most cases for eight males and sixteen females in each group except in the stock rats for which they are the means of twenty-three males and twenty-three females which were average rats picked from twenty-three successive litters.[4]

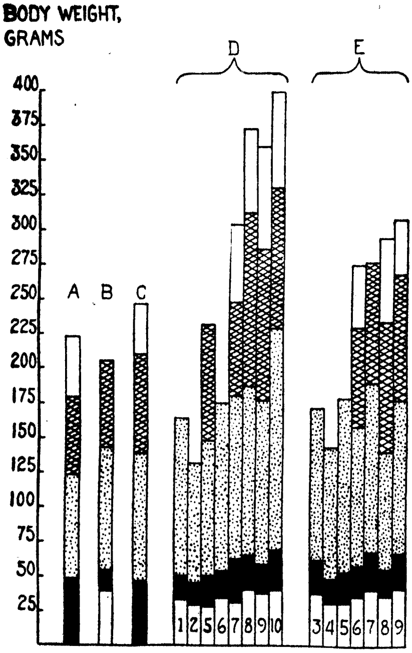

FIGURE I

Fig. 1 The body weight of male rats at 21 days (to top of lower blank space), at 30 days (to top of solid block), at 60 days (to top of stippled area), at 90 days (to top of cross-hatched area), and at 120 days (to top of upper blank space). The following letters designate the groups: A — Donaldson ('24); B — stock rats, University of Southern California; C — Sherman and Campbell ('24) on diet B; D — present tests with first litter rats; E — present tests with second litter rats. Figures in lower blank spaces in D and E indicate successive generations.

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[3] The stock diet (diet 2) had the following percentage composition: whole ground yellow corn, 45.0; whole wheat flour, 28.5; powdered skimmed milk, 18.0; alfalfa leaf meal, 4.0; brewer's yeast (Anheuser-Busch, Strain K), 2.0; cottonseed oil with vitamins A and D (6 parts cottonseed oil and 1 part superbiotol; mixture has 430 I.U. vitamin A and 60 I.U. vitamin D per pound), 1.5%; $CaCO_3$ (powdered), 0.5; and NaCl (commercial), 0.5.

[4] We wish to thank Miss Elizabeth G. Sumner who determined these values.

A summary of the data obtained from the breeding trials and pertinent results with respect to the litters are included in table 2.

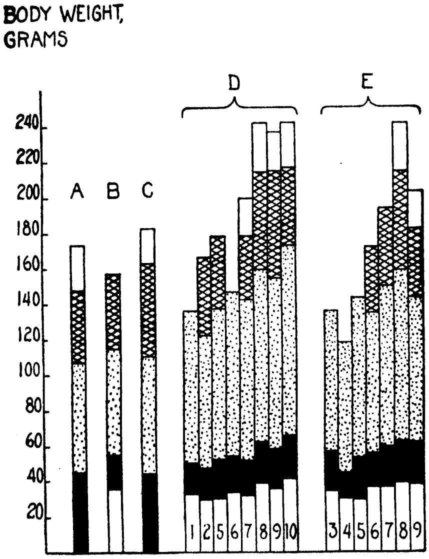

FIGURE II

Fig. 2 The body weight of female rats at 21 days (to top of lower blank space), at 30 days (to top of solid block), at 60 days (to top of stippled area), at 90 days (to top of cross-hatched area), and at 120 days (to top of upper blank space). The following letters designate the groups: A — Donaldson ('24); B — stock rats, University of Southern California; C — Sherman and Campbell ('24) on diet B; D — present tests with first litter rats; E — present tests with second litter rats. Figures in lower blank spaces in D and E indicate successive generations.

DISCUSSION

The results of the present experiments strongly support the view of Sherman and Campbell that diets satisfactory for growth and reproduction may not be optimal. Our stock diet (diet 2), which has been used successfully for 12 years with many generations in our stock colony, gives a rate of growth distinctly inferior to that obtained with the modified Sherman diet B (our diet 57) where the butterfat was replaced by a margarine fat enriched with vitamin A. The weight of male rats at 90 days of age on our stock diet averaged 205 gm. while the weights of the tenth generation of first litter rats on diet 57 had a mean average weight of 330 gm.; with the second litter rats on diet 57, the average value for the ninth generation was 267.5 gm. The growth also exceeds that obtained with rats of the Osborne and Mendel strain by Sherman and Campbell ('24) for males on their diet B (210.4 gm.) as well as the values of Donaldson ('24) in which the average weight is

TABLE 2

Summary of data obtained from breeding trials with successive generations of rats raised on diet 57 (a modified Sherman Diet B).

PARENTS					LITTER						
Generation	Females bred	Litters born	Average period after mating [1]	Average age of mother at birth of litter	Generation	Average number per litter	Average weight per rat		Number of rats weaned	Number of rats died	Number of litters destroyed [3] or stillborn
							At birth (*) or 3 days	At 21 days [2]			
First litter rats											
			days	*days*			*gm.*	*gm.*			
0	7	7	25.1	....	1	10.7	5.00*	31.1(35)	45	3	0
1	8	8	24.3	108.0	2	11.5	5.22*	29.3(42)	55	1	0
2	8	8	27.8	115.5	3	9.0	5.57*	......	..	..	..
3	13	13	25.6	112.7	4	11.9	5.00*	28.4(77)	79	0	0
4	8	7	26.7	122.7	5	9.9	4.87*	27.6(35)	39	12	0
5	8	8	26.2	120.4	6	11.2	5.19*	32.4(56)	56	0	0
6	8	7	25.9	148.7	7	11.2	5.80	31.3(35)	38	4	1
7	8	6	26.0	149.0	8	9.8	8.02	39.3(35)	35	0	1
8	10	5	25.0	145.5	9	10.6	7.00	36.6(35)	35	0	0
9	14	12	28.0	149.8	10	8.4	6.88	39.2(42)	70	3	1
10	14	13	30.8	152.5	11	8.4	7.91	36.6(63)	75	0	2
Second litter rats											
2 [4]	8	8	27.8	176.5	3	7.8	5.99*	31.2(28)	39	7	0
3	8	8	24.4	171.5	4	12.1	5.35*	28.5(42)	58	0	0
4	8	8	25.3	191.3	5	10.4	4.99*	27.3(35)	47	14 [5]	0
5	8	5	31.8	185.2	6	10.4	6.76	31.6(7)	28	4	0
6	7	6	27.5	216.9	7	7.4	7.89	35.2(21)	29	3	1
7	8	7	25.0	197.5	8	8.7	7.83	34.1(35)	40	0	0
8	10	10	26.4	213.5	9	9.1	8.04	36.4(35)	60	8	0

[1] Period after male was placed in cage.

[2] Figures in parentheses are number of rats of which this value is the average. Only those litters where there were 7 in a litter at 21 days are included.

[3] Destroyed before 3 days.

[4] Second litter of first litter parents. The other rats in this group are from second litters of second litter parents.

[5] Includes 6 stillborn in one litter of 12 and 1 stillborn in a litter of 7.

184.8 gm. However, they fall far short of the maximum growth rate attainable in the Osborne and Mendel rats as reported by Anderson and Smith ('32). Similar variations in the weights of the female rats were observed, the 90-day averages being as follows: stock diet 2, 157 gm.; diet 57, tenth generation of first litter rats, 216 gm., and ninth generation of second litter rats, 182 gm. For comparison, the figures obtained by Sherman and Campbell for rats on diet B were 162.8 gm., and the values of Donaldson were 148 gm. However, because of differences in rate of growth not only of different strains of rats but also of different stock colonies of the same strain, it is not possible to make quantitative comparisons between different laboratories. Nevertheless, it would appear that our diet 57 is superior to our stock diet 2 to about the same extent that diet B excelled diet A in the Sherman-Campbell tests.

The number of females failing to give birth to young was 11% in the first litter group and 9% in the second litter rats. If one includes in this calculation the females whose litters were destroyed before 3 days, the figures become 16 and 11%, respectively, values comparing favorably with the 19% reported by Sherman and Campbell for their diet B in contrast to their value of 48% for diet A.

The results show a somewhat downward trend with respect to the weight at 21 days and the growth rate through the fourth generation and the marked improvement that followed, so that the results for the tenth generation of first litter rats and for the ninth generation of the second litter rats are the best of any group. The preliminary slump may have been due to the use of the commercial whole wheat flour which was later replaced by ground whole wheat prepared in our laboratory as needed.

It would also appear that the rate of growth is better in first litter rats than in the second litter although the 3-day and 21-day values are identical in the last litters reported in table 2. There also would seem to be a progressive improvement in weight of the first litter rats in the later generations. This may, in part, be traceable to selection of the more vigorous stock for breeding rather than as a result of diet. However, the growth studies in each generation were carried out on animals from all litters; in the later generations all of these were bred.

These results answer in the affirmative the question raised by Boutwell et al. ('43) as to the adequacy of a vegetable fat for continued growth and reproduction over a number of generations. Thus, a diet which contained no more butterfat than that present in skimmed milk powder to which was added as the main fat a vegetable margarine in the proportion of fat found in whole milk powder, and which was supplemented once weekly with 5 gm. each of lean beef and lettuce, effectively supported growth and reproduction over ten generations.

SUMMARY

Experiments are reported on reproduction and growth rate of ten generations in which the lineage is through the first litter and of eight generations in which the lineage is through the second litters where the diets have been a modification of the Sherman diet B in which butterfat has been replaced by vitamin A-fortified margarine fat. The growth rate considerably exceeded that obtained with animals on our stock diet and progressively improved with the later generations. Somewhat faster growth was obtained with the first litter rats than with those in the second litter group. It is concluded that a vegetable fat such as that contained in a margarine can serve adequately in place of butterfat for growth and reproduction on a diet otherwise nutritionally satisfactory.

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COMPOSITION OF TYPICAL MEXICAN FOODS[1]

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A nutrition program cannot be properly planned in any country until data on the composition of its foods are at hand. On the basis of these data one may select foods which will integrate to form a nutritionally balanced menu at the lowest cost.

In preparing for a school lunch demonstration in Mexico City, it was first necessary to undertake the analysis of the major foods of Mexico because the few analyses that had been reported by Gavarre ('40), Gonzalez ('42), Alvarez ('42) and Zozaya and Alvaredo ('43) were incomplete. The compilation of data by Axtmayer and Cook ('42) indicates that little is known of the composition, especially the vitamin content, of Latin American foods.

In the present study 112 samples of food from Mexico were analyzed for carotene, thiamine, riboflavin, niacin, ascorbic acid, calcium, phosphorus, iron, nitrogen, ash and total solids content. To our knowledge many of these foods were analyzed for the first time. The seed and legume samples were harvested in 1942 and 1943, while most of the remaining samples were grown during the first half of 1944.

EXPERIMENTAL

1. *Method of sampling and shipping*

The fruit and vegetable samples were purchased in the public markets, usually in Mexico City, packed in waxed cartons, imbedded in carbon

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[2] The National Institute of Nutrition of Mexico.

[3] The Massachusetts Institute of Technology, Cambridge.

[4] The International Health Division, Rockefeller Foundation, Mexico City.

dioxide ice and while frozen shipped by air express to Boston. Preliminary tests by Lockhart et al. ('44) had shown no measurable vitamin losses during 4 days' storage under these conditions. All samples arrived within 3, and usually 2 days and were held in a carbon dioxide ice refrigerator until analyzed. The seed and legume samples were shipped by parcel post in cardboard or wooden cartons, and were stored in glass jars at room temperature until analyzed.

A portion of each sample was boiled in 3% metaphosphoric acid, macerated in a Waring Blendor, and analyzed immediately for ascorbic acid content. The remainder of each sample was macerated without pre-treatment, returned to the carton, and refrozen. Portions of this frozen sample were taken as required, but all vitamin analyses on a sample were made during the same day.

2. *Methods of analysis*

The A.O.A.C. ('40) spectrophotometric method was used to determine the carotene content of all samples except the dry chilis, for which the chromatographic procedure of Moore ('40) was used. Thiamine was measured according to Moyer and Tressler ('42), riboflavin according to Andrews ('43), niacin according to the U. S. Pharmacopeia ('43) and ascorbic acid by the Hochberg, Melnick and Oser ('43) modification of the method by Bessey and King ('33). Moisture, ash, nitrogen, calcium and manganese content were measured according to the A.O.A.C. ('40), phosphorus according to Fiske and Subba-Row ('25) and iron according to Koenig and Johnson ('43).

The results of these analyses are presented in tables 1 to 4. The foods have been arranged alphabetically according to the Spanish name. The English translation is given whenever it is known. These data are presented on the "wet basis", in terms of the foods as received, because they should be more useful in this form. The composition on the "dry basis" may easily be calculated.

DISCUSSION

The analyses of some of these foods do not always agree with previous reports in the scientific literature. Though the samples were carefully taken, and all shipments were received in excellent condition, the food may have been improperly handled previous to collection. Also, the difficulties inherent in sampling ½ pound amounts of a vegetable or fruit are great. That several samples must be obtained from several areas before one can be satisfied with the data on each food is indicated by the results on six separate samples of malva (table 1). The considerable variation in the content of certain nutrients indicates that differences in botanical strain, in soil and in climate may affect the nutrient value of foods.

Food data are no more accurate than the methods of analysis used to obtain them. While the most acceptable chemical and physical methods were used in this investigation, there was some inaccuracy due to interference by chemical substances present in plant tissue. This interference may be especially misleading when a species of plant is analyzed for the first time.

1. *Protein*

No attempt has been made to calculate the protein content from the nitrogen values because the correct conversion factor is not yet known for many of these foods.

If the usual factor (N × 6.25) were to be employed, at least ten of the foods contained over 22% protein. The foods richest in nitrogen content (expressed as gm./100 gm.) were: charales 9.9, parota 5.9, India squash seed 5.2, Castilla squash seed 5.0, peanut 4.4, guaje seed 4.2, sesame seeds 3.9, piñón 3.6, lentil 3.6, lima bean 3.6, palacio bean 3.6 and bayo gordo bean 3.6. Undoubtedly, the quality of the proteins of these foods differs. However, most are legumes, and legume proteins are generally superior to cereal proteins.

2. *Calcium*

A number of these foods were rich in calcium content, the most important (in mg./100 gm.) were: charales 4160, sesame seed 417, guaje seed 322, epazote 260, malva 257, bayo gordo bean 204, chipotle chile 195, leek 190, tuna roja 181, cocona bean 180, black bean 160, palacio bean 159, small alubia bean 157, hediondilla 155 and morita chili 153. The calcium in these foods may not be equally available. Charales were high in calcium primarily because of the skeletal tissue content. The unusually high quantity of calcium in malva deserves attention for, unlike many foods in this group, it is relatively easy for one to consume 100 gm. as a daily portion.

3. *Phosphorus*

The fourteen foods richest in phosphorus content (in mg./100 gm.) were: charales 2640, Castilla squash seed 834, India squash seed 655, piñón 588, sesame seed 566, parota 544, lima bean 439, guaje seed 411,

TABLE 1

Composition of vegetables.

ENGLISH NAME	SPANISH NAME	BOTANICAL NAME	CULTIVATED IN	CONTENTS PER 100 GM. AS RECEIVED										
				Water	Nitrogen	Ash	Calcium	Phosphorus	Iron	Carotene	Thiamine	Riboflavin	Niacin	Ascorbic acid
				gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Chard	Acelga	Beta vulgaris, var. cycla	D. F.	89.3	0.52	2.4	71	36	2.5	4.35	0.06	0.21	0.68	8.0
Watercress	Berro	Nasturtium aquaticum L.	Morelos	93.3	0.39	1.3	122	59	2.4	1.04	0.07	0.13	1.04	3.2
Beets	Betabel	Beta vulgaris, var. rapacea	D. F.	82.3	0.53	1.0	20	73	1.3	0.00	0.05	0.05	0.38	29.4
Squash, small	Calabacitas	Cucurbita mexicana L.	Morelos	92.6	0.29	0.7	14	53	16.4	0.29	0.08	0.08	0.56	8.8
Squash blossoms	Calabacitas, flor de	Cucurbita mexicana L.	D. F.	92.2	0.28	1.2	29	167	1.3	0.94	0.16	0.20	0.98	3.9
Potato, sweet	Camote	Ipomoca batatas L.	Morelos	60.7	0.10	0.8	39	27	1.2	0.45	0.09	0.03	0.28	21.3
....	Chayote con espinas	Sechium edule, Swartz	Morelos	92.1	0.13	0.4	7	26	0.4	0.03	0.03	0.02	0.22	25.2
....	Chayote sin espinas	Sechium edule, Swartz	Veracruz	88.6	0.16	0.6	27	34	1.0	0.00	0.03	0.07	0.43	7.6
Peas, green	Chicharos	Pisum sativum, L.	México	75.6	1.28	0.7	27	97	2.3	0.57	0.33	0.10	2.33	67.0
....	Chinchayote	Sechium edule Sw.		74.6	0.33	1.0	13	68	1.7	0.01	0.01	0.04	1.53	10.4
(Root of chayote)	Chilacayote	Cucurbita filicifolia Bouch		94.2	0.20	0.5	22	28	0.6	0.06	0.03	0.08	0.38	1.9
Coriander	Cilantro	Coriandrum sativum, L.	D. F.	88.6	0.51	1.6	110	45	2.0	4.31	0.13	0.02	0.99	10.6
Cabbage	Col	Brassica oleracea, L. var. capitata	D. F.	89.6	0.37	0.6	29	42	1.1	0.14	0.11	0.06	0.40	20.4
Cauliflower	Coliflor	Brassica oleracea, L. var. botrytis	D. F.	90.0	0.45	1.0	33	66	3.7	0.05	0.15	0.10	0.69	153.7
Beans, green	Ejote	Phaseolus vulgaris	Morelos	88.7	3.41	0.7	51	49	2.5	0.42	...	0.09	0.50	11.5
Corn, fresh white	Elotes, blanco	Zea mays		65.7	0.45	0.7	32	44	1.2	0.00	0.16	0.09	1.52	99.7
....	Endivia	Sonchus oleraceus	Hidalgo	92.0	0.39	1.7	93	35	12.5	4.99	0.07	0.12	0.43	4.7
Chenopodium	Epazote	Chenopodium ambrosioides	Morelos	89.6	0.46	2.2	260	39	5.4	1.02	0.04	0.13	0.57	3.7

....	Jicama	Pachirhizus palmatilobus	Morelos	91.8	0.07	0.3	11	13	0.5	0.03	0.02	0.01	0.11	13.7
Lettuce	Lechuga	Lactuca sativa, L.	D. F.	96.6	0.23	0.6	31	30	1.2	0.61	0.11	0.06	0.25	3.0
....	Lengua de vaca	Rumex crispus L.	D. F.	93.0	0.52	1.8	82	69	6.4	4.10	0.09	0.13	0.20	...
				93.0[1]	0.25	1.2	93	29	5.2	1.33	0.07	0.19	0.61	31.7
Century plant flower	Maguey, flor de	Agave atrovirene	Hidalgo	91.0	0.15	0.6	114	27	0.9	0.43	0.11	0.05	0.21	58.6
Malva	Malva	Malva parviflora	Hidalgo	85.9	0.94	2.6	312	73	19.5	2.53	0.11	0.19	0.95	45.4
		Malva parviflora	D. F.	92.4	0.34	2.2	268	42	9.2	2.40	0.07	0.17	0.51	...
		Malva vastrum peruvianum	D. F.	91.2	0.40	2.2	292	29	8.7	4.31	0.07	0.22	0.45	...
		Malva silvestris	D. F.	92.0	0.33	1.7	221	36	9.7	4.80	0.10	0.24	0.44	...
		Malva parviflora	D. F.	82.9	0.96	2.9	188	111	11.3	8.50	0.16	0.26	1.24	14.6
		Modiola multifida M.	D. F.	91.5	0.35	3.6	263	39	6.0	2.13	0.08	0.16	0.38	...
....	Mezquite	Prosopis juliflora L.	Hidalgo	71.5	1.02	1.0	140	57	0.1	0.15	0.05	0.05	0.95	...
Turnip, flower	Nabo, flor de	Brassica campestris	D. F.	87.4	0.67	1.5	117	97	3.4	3.66	0.04	0.02	1.21	41.6
Turnip, leaves	Nabo, hojas de	Brassica campestris	D. F.	84.0	0.58	1.0	138	38	5.2	2.84	0.06	0.13	0.52	147.3
Cactus leaf	Nopales	Opuntia hyptiacantha	Mexico	92.5	0.21	1.0	105	27	0.8	0.50	0.04	0.04	0.30	4.1
Cucumber	Pepino	Cucumis sativus	D. F.	95.4	0.12	0.5	18	32	0.5	0.11	0.03	0.04	0.18	17.1
Parsley	Perejil	Carum petroselium Benth.	D. F.	84.8	0.65	1.9	152	55	4.1	4.56	0.16	0.37	1.95	12.1
Leek	Porro	Allium porrum L.	D. F.	71.5	0.20	0.7	40	46	1.0	0.05	0.10	0.06	0.39	6.3
....	Quelites	Amaranthus chlorostachys	Morelos	86.1	0.64	3.0	190	39	4.6	4.60	0.12	0.19	0.57	67.9
....	Romerito	Dondia sufrutencona	D. F.	92.0	0.58	1.8	41	18	2.5	2.76	0.12	0.08	0.30	4.4
....	Tejocotes	Crataegus mexicana Moc. et Sess	D. F.	76.1	0.15	0.7	91	37	1.2	6.40	0.05	0.05	0.48	18.6
Purslane	Verdolagas	Portulaca oleracea, L.	Morelos	90.7	0.36	1.9	86	40	5.2	3.25	0.08	0.11	0.56	10.8
....	Xocoyol	Oxalis divergens	Hidalgo	91.0	0.68	0.9	79	38	5.1	3.70	0.12	0.12	0.42	26.3
				92.6	0.67	0.8	182	32	3.5	3.34	0.11	0.15	0.48	...
Yucca flower	Yuca, flor de	Yucca alcifolia, L.	Hidalgo	90.0	0.49	0.7	47	73	0.5	0.02	0.14	0.09	0.58	11.7
Carrot	Zanahoria	Daucus carota L.	D. F.	91.1	0.08	0.6	20	30	0.0	5.14	0.05	0.03	0.30	26.8

[1] Ground with moisture added. Sample used for all analyses except ascorbic acid.

TABLE 2

Composition of fruits.

ENGLISH NAME	SPANISH NAME	BOTANICAL NAME	CULTIVATED IN	CONTENTS PER 100 GM. AS RECEIVED										
				Water	Nitrogen	Ash	Calcium	Phosphorus	Iron	Carotene	Thiamine	Riboflavin	Niacin	Ascorbic acid
				gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Avocado	Aguacate	Persea gratissima L.	Morelos	79.7	. . .	0.8	16	47	1.03	0.18	0.07	0.10	0.80	33.3
Wild cherry	Capulín	Prunus capuli L.	D. F.	81.2	0.18	0.6	18	24	0.72	0.51	0.03	0.03	0.65	8.4
Apricots	Chavacono	Prunus armeniaca, L.	Puebla	83.4	0.09	0.8	17	32	1.20	2.54	0.03	0.03	0.41	2.5
Custard apple	Chirimoya	Annona cherimolla	D. F.	30.6	0.42	0.8	23	43	0.41	0.02	0.13	0.15	2.03	6.8
Plums	Ciruelas	Spondias purpurpea L.	Guerrero	86.5	. . .	0.4	44	24	0.94	0.38	0.05	0.02	0.26	45.5
Strawberry	Fresa	Fragaria mexicana	Gto.	91.0	0.13	0.5	28	30	2.12	0.06	0.03	0.03	0.28	27.8
. . . .	Granada China	Passiflora ligularis	Puebla	78.5	0.44	1.4	53	71	1.27	0.25	0.01	0.10	1.51	2.0
Guavas	Guayaba	Psidium guayava L.	Morelos	81.5	0.15	0.7	30	29	0.70	1.47	0.06	0.04	1.20	89.4
Figs	Higo	Ficus carica	D. F.	83.0	0.21	0.8	65	22	0.38	0.13	0.05	0.05	0.44	3.6
Tomato	Jitomate	Lycopersicum esculentum	D. F.	94.6	0.12	0.5	6	22	0.36	3.54	0.06	0.03	0.37	17.9
. . . .	Lima	Citrus limetta	Guerrero	91.7	0.09	0.3	16	15	0.21	0.02	0.04	0.02	0.14	51.0
. . . .	Limón agrio	Citrus limonia O.	Veracruz	88.4	0.15	0.5	5	27	0.66	0.04	0.05	0.03	0.20	18.6
. . . .	Limón real	Citrus médica	Puebla	90.3	0.09	0.4	25	24	0.21	0.04	0.12	0.02	0.33	28.1
. . . .	Mamey	Calocarpum mamosum	Puebla	71.4	0.18	1.6	36	38	0.37	1.46	0.03	0.00	2.14	22.7
Tangerine	Mandarina	Citrus nobilis var. deliciosa Swingle	San Luis Potosi	84.8	0.16	0.4	69	22	0.37	1.81	0.10	0.03	0.22	111.8
Manila mango	Mango (Manila)	Mangifera indica var. manila	Oaxaca	79.6	0.12	0.4	7	16	0.23	1.96	0.14	0.05	1.64	171.1
Common mango	Mango (Corriente)	Mangifera indica	Oaxaca	83.2	0.11	0.4	19	6	0.30	1.17	0.01	0.02	0.21	33.3
Muskmelon	Melón	Cucumis melo L.	Morelos	93.5	0.10	0.5	15	11	0.79	1.68	0.03	0.02	0.53	23.6
Orange	Naranja	Citrus aurantium	Veracruz	86.6	0.13	0.4	33	17	0.20	0.29	0.09	0.03	0.25	182.3
Papaya	Papaya	Carica papaya L.	Veracruz	91.2	0.09	0.6	36	23	0.37	1.41	0.02	0.03	0.28	64.8
. . . .	Perón	Pyrus malus	Puebla	83.4	0.04	0.5	9	15	0.66	0.02	0.04	0.02	0.15	. . .
Pineapple	Piña	Ananas nativum	Veracruz	88.5	0.08	0.3	13	5	0.41	0.18	0.11	0.03	0.18	41.1
Banana	Platano macho	Musa paradesiaca	Veracruz	78.6	. . .	0.8	10	34	0.55	0.28	0.03	0.05	0.65	10.6
Banana	Platano tabasco	Musa sapientum	Chiapas	60.2	. . .	0.8	7	40	0.93	1.95	0.05	0.02	0.52	17.5
. . . .	Tomate	Physalis aequata	Morelos	92.5	0.22	0.4	9	33	0.70	0.19	0.07	0.03	0.66	76.4
. . . .	Tuna, blanca	Opuntia hyptiacantha	Hidalgo	86.2	. . .	0.4	36	34	0.50	0.08	0.01	0.02	. . .	. . .
. . . .	Tuna, colorada	Opuntia robusta	Hidalgo	82.0	. . .	0.7	63	32	0.70	0.12	0.01	0.02	. . .	. . .
. . . .	. . . .		Hidalgo	89.3	0.10	1.3	181	11	0.93	0.01	0.01	0.02	0.24	19.1
. . . .	Xoconoxtle	Opuntia imbricata	Hidalgo	83.5	. . .	0.7	110	14	0.50	0.05	0.45	0.23	0.15	21.9
Sapote, white	Zapote, blanco	Casimiroa edulis	Puebla	89.3	0.25	0.4	8	19	0.23	0.03	0.03	0.06	1.00	15.7
Sapote, small	Zapote, chico	Achras zapota L.	Veracruz	78.5	0.09	0.5	22	6	0.63	0.05	0.02	. . .	0.24	6.3
			Morelos	83.1	0.07	0.6	22	23	0.26	0.19	0.00	0.02	0.20	191.7

TABLE 3

Composition of seeds and legumes.

ENGLISH NAME	SPANISH NAME	BOTANICAL NAME	CULTIVATED IN	CONTENTS PER 100 GM. AS RECEIVED										
				Water	Nitrogen	Ash	Calcium	Phosphorus	Iron	Carotene	Thiamine	Riboflavin	Niacin	Ascorbic acid
				gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Sesame seed	Ajonjolí	Sesamum indicum L.	Guerrero	3.8	3.88	4.4	417	566	8.4	0.02	1.36	0.23	5.01	0.5
Bean	Alubia Chica	Phaseolus vulgaris L.	Querétaro	9.2	3.23	3.7	157	406	6.6	0.01	0.11	0.18	1.82	3.1
Bean	Alubia grande	Phaseolus vulgaris L.	Querétaro	11.4	3.21	3.7	108	362	6.6	0.05	0.81	0.20	2.14	3.0
Rice	Arroz	Oriza sativa L.	Morelos	12.0	1.40	0.8	8	172	0.8	0.00	0.32	0.03	2.40	0.0
Peas (dried)	Alverjon	Pisum sativum	Puebla	10.2	3.27	3.1	72	287	7.5	0.02	0.91	0.18	2.30	0.0
Oats	Avena	Avena sativa L.	México	6.3	2.58	1.5	61	278	3.3	0.00	0.53	0.11	0.82	0.0
Peanut	Cacahuate	Arachis hipogea L.	Guanajuato	5.1	4.40	2.0	49	292	2.1	0.01	0.95	0.13	14.42	0.7
Sugar cane	Caña	Saccharum officinarum var. F. C916	Morelos	74.4	0.04	0.3	21	10	0.6	0.00	0.36	0.01	0.10	0.0
Beans, ayocote	Frijol ayocote	Phaseolus vulgaris L.	Guanajuato	11.7	2.39	3.6	116	262	5.9	0.03	0.42	0.19	1.92	1.3
Bean, bayo gordo	Frijol bayo gordo	Phaseolus vulgaris L.	Zacatecas	10.1	3.56	3.6	204	291	5.5	0.04	0.75	0.22	1.19	0.9
Bean, cocona	Frijol cocona	Phaseolus vulgaris L.	Puebla	10.9	3.27	3.6	155	382	11.5	0.00	0.82	0.15	1.37	1.4
			Guanajuato	9.5	3.28	3.8	206	320	7.1	0.02	1.14	0.17	1.64	3.3
Bean, black	Frijol negro	Phaseolus vulgaris L.	Puebla	13.7	3.11	3.4	142	382	8.7	0.04	0.81	0.22	1.69	1.1
			Querétaro	9.5	3.26	3.6	177	345	7.3	0.01	0.96	0.18	1.86	4.4
Bean, palacio	Frijol palacio	Phaseolus vulgaris L.	Guanajuato	10.6	3.55	3.3	159	369	6.9	0.03	0.85	0.13	1.57	2.1
Pea, chick (dried)	Garbanzo breve	Cicer arientium L.	Sonora	9.1	3.44	3.4	100	403	9.3	0.08	0.84	0.18	1.67	1.3
			Sonora	7.7	3.27	3.4	110	399	8.4	0.05	0.65	0.16	1.41	0.0
Bean, lima	Haba	Faba vulgaris, M vicia faba L.	Puebla	8.9	3.63	3.2	49	439	7.3	0.08	0.91	0.31	2.30	4.9
Lentil	Lenteja	Ervum lens L.	Querétaro	11.2	3.62	2.5	82	307	5.4	0.09	0.73	0.21	1.96	7.5
			Querétaro	9.4	3.56	3.0	86	404	5.8	0.90	0.67	0.16	1.51	3.2
Corn, white	Maíz	Zea mays L.		12.2	1.29	1.2	7	253	2.5	0.03	0.32	0.08	1.58	1.3
			Edo. Mexico	10.5	1.19	1.3	10	261	2.8	0.03	0.42	0.09	1.70	1.9
....	Parota	Enterolobium ciclocarpum	Morelos	1.4	5.88	3.2	29	544	4.2	0.04	2.72	0.21	1.75	6.6
....	Piñón	Pinus edulis Eng. P. cembroides		2.3	3.59	2.9	13	588	5.2	0.02	1.11	0.24	5.29	0.9
Seed of Castilla squash	Semilla de Calabaza de Castilla	Cucurbita pepo L.	Morelos	4.9	4.96	5.1	39	834	11.0	0.05	0.26	0.17	2.30	0.0
Seed of India squash	Semilla de Calabaza de India	Cucurbita pepo L.	Morelos	4.2	5.17	4.9	37	655	8.9	0.06	0.36	0.23	3.28	0.1
....	Semilla de Guaje	Leucanea suculenta L.	Morelos	7.8	4.22	5.0	322	411	15.2	2.25	1.44	0.21	4.00	31.3
Wheat	Trigo	Triticum vulgare L.	Guanajuato	10.1	1.56	1.7	50	290	9.2	0.01	0.44	0.10	3.68	0.0

TABLE 4

Composition of chilis and chili seed (Semilla) and miscellaneous foods.

ENGLISH NAME	SPANISH NAME	BOTANICAL NAME	CULTIVATED IN	CONTENTS PER 100 GM. AS RECEIVED										
				Water	Nitrogen	Ash	Calcium	Phosphorus	Iron	Carotene	Thiamine	Riboflavin	Niacin	Ascorbic acid
Chili, dry	Chile seco			*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
	Ancho	Capsicum annum L. grossum Sendt	Aguascalientes	10.0	1.57	6.0	70	212	5.7	38.94	0.16	0.71	3.44	143.0
	Semilla			6.5	2.97	3.7	32	446	7.3	0.20	1.28	0.15	8.20	64.8
	Cascabel	Capsicum annum L. cerasiforma Mill.	Jalisco	9.5	1.67	7.5	75	216	4.7	18.60	0.14	0.54	9.70	58.8
	Semilla			7.4	2.42	2.9	42	472	5.3	0.40	0.91	0.18	10.20	44.1
	Chipotle	Capsicum annum L. dulce Hort	Hidalgo	16.1	1.95	6.6	195	281	6.1	6.05	0.33	0.62	13.00	24.2
	Semilla			8.3	2.94	3.8	68	517	6.2	0.26	0.93	0.35	9.80	40.5
	Guajillo	Capsicum annum L. longum Sendt	Aguascalientes	17.6	1.68	7.2	91	189	10.1	44.40	0.16	0.76	4.50	135.0
	Semilla			5.6	2.60	3.3	44	476	7.1	0.45	1.92	0.21	11.10	34.4
	Morita	Capsicum annum L. abreviatum Fing	Puebla	14.0	2.28	7.1	153	157	5.2	10.50	0.23	1.37	15.40	98.6
	Semilla			9.2	2.87	3.7	79	457	5.6	0.51	2.23	0.50	11.29	209.0
	Mulato	Capsicum annum L. grossum Sendt	Puebla	15.3	1.43	5.0	80	199	12.8	39.00	0.29	0.60	4.09	111.5
	Semilla			6.8	2.64	3.4	45	492	8.3	0.40	2.64	0.19	7.80	36.4
	Pasilla	Capsicum annum L. longum Sendt	Jalisco	15.9	1.72	6.2	105	163	6.3	56.60	0.43	1.00	7.84	54.3
	Semilla			5.8	2.63	3.0	37	422	7.4	0.12	1.28	0.23	9.50	56.3
	Piquin con semillas	Capsicum frutescens L. var. baccalum	Hidalgo	9.5	2.27	4.0	127	320	7.8	4.50	0.56	0.44	15.20	71.1
Chili, fresh	Chile verde													
	Jalapeño	Capsicum annum L.	Veracruz	92.1	0.22	0.5	12	29	0.5	0.33	0.05	0.03	0.65	75.2
	Mulato	Capsicum annum L. grossum Sendt	Tamaulipas	89.2	0.32	0.6	17	35	1.4	1.18	0.17	0.06	1.10	174.5
	Serrano	Capsicum annum L. acuminatum Fing	Veracruz	86.8	0.43	0.8	25	54	0.8	0.55	0.11	0.06	1.52	57.9
Miscellaneous	Agua miel		Hidalgo	87.8	. . .	. .	10	20	0.4	0.00	0.10	0.01	0.50	11.3
	Charales		D. F.	3.9	9.9	. .	4160	2640	22.7	3200[1]	0.40	0.10	6.00	0.0
	Gusanos	Hipopta agavis	Hidalgo	66.9	1.8	. .	. . .	. . .	. . .	0.05	0.49	0.37	2.40	3.9
	Pulque		D. F.	97.0	0.7	. .	10	10	0.7	0.00	0.20	0.02	0.30	6.2
	Quesa de tuna		D. F.	17.2	0.2	. .	51	29	13.0	0.10	0.20	0.20	1.50	88.0
	Tortilla			38.9	0.8	1.0	131	195	2.8	0.19	0.18	0.05	0.89	0.0
				43.2	1.0	0.9	101	178	1.9	. . .	0.21	0.07	1.07	. .
				43.7	0.9	0.9	101	178	1.8	. . .	0.19	0.06	0.93	. .

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[1] I.U. of vitamin A.

small alubia bean 406, chick pea 401, palacio bean 369, black bean 363, large alubia bean 362 and cocona bean 351.

4. *Calcium: phosphorus ratio*

Nutritionally, foods rich in phosphorus content may be undesirable, especially for populations like the Mexican, whose intake of calcium and vitamin D may be limited. It is generally agreed that a calcium: phosphorus ratio of approximately 1:1 is best in the human dietary. The calcium: phosphorus ratios of those foods rich in either of these minerals were as follows: epazote 6.7, malva 4.7, charales 1.58, morita chili 0.97, guaje seed 0.78, sesame seed 0.74, bayo gordo bean 0.70, chipotle chili 0.69, cocona beans 0.46, black bean 0.44, palacio bean 0.43, small alubia bean 0.39. The Ca: P ratio of large alubia beans, chick pea, lima bean, India squash seed, Castilla squash seed, parota and piñón were each 0.30, or below. Thus it is evident that epazote, malva and charales are foods which may be especially useful for calcium and phosphorus metabolism.

5. *Iron*

The foods highest in iron content (in mg./100 gm.) were: charales 23, calabacitas 16, guaje seed 15, quesa de tuna 13, mulato chili 13, endivia 13, cocona bean 12, malva 11, Castilla squash seed 11, hediondilla 9, India squash seed 9, wheat 9 and chick peas 9. There are few foods in the American dietary as rich as these in iron content. The nutritional availability of the iron in Mexican foods has not yet been determined.

6. *Carotene*

The carotene content of the samples of dry chili ranged between 5 and 57 mg. (equivalent to 8200 and 95000 I.U. of vitamin A) per 100 gm. Only 6 gm. of pasilla chili would be needed to supply the entire daily allowance advised by the Food and Nutrition Board ('43) for an adult man. It is evident that chili can be an important source of provitamin A in the Mexican dietary. Other foods rich in carotene (expressed as mg./100 gm.) were: tejocote 6, carrot 5, endivia 5, quelites 5, parsley 5, lengua de vaca 5, chard 4, cilantro 4, spinach 4, malva 4, coriander 4, turnip flower 4 and jitomate 4. All of these contain at least 7000 I.U. of vitamin A per 100 gm., assuming a direct conversion factor for carotene.

7. *Thiamine*

The foods richest in thiamine content (in mg./100 gm.) were: parota 2.7, chili seed 1.3 to 2.6, guaje seed 1.4, sesame seed 1.4, piñón 1.1, peanut 1.0, cocona bean 1.0, lima bean 0.9 and alverjon 0.9. The high content of thiamine in parota is notable.

8. *Riboflavin*

None of these foods was rich in riboflavin content. Dry chili contained 0.4 to 1.4 mg. per 100 gm. but a day's portion of chili can hardly be considered a significant source. The other foods highest in this vitamin (in mg./100 gm.) were: gusanos 0.4, parsley 0.4, lima bean 0.3, spinach 0.3, hediondilla 0.3, piñón 0.2, India squash seed 0.2, xoconoxtle 0.2, sesame seed 0.2, bayo gordo bean 0.2, black bean 0.2, quesa de tuna 0.2, guaje seed 0.2, alubia bean 0.2, malva 0.2, and chard 0.2.

9. *Niacin*

Though some chilis contained much niacin, peanuts were the most important source of this vitamin. The foods richest in niacin (as mg./100 gm.) were: dry chili 3.5 to 15.4, peanuts 14.4, charales 6.0, piñón 5.3, sesame seed 5.0, guaje seed 4.0, wheat 3.7, India squash seed 3.3, gusanos 2.4, rice 2.4, Castilla squash seed 2.3, green peas 2.3, alverjon 2.3, lima bean 2.3, mamey 2.1, large alubia 2.1 and custard apple 2.0.

10. *Ascorbic acid*

Several foods were exceptionally rich in this vitamin. The contents (mg./100 gm.) found were: black sapote 192, orange 182, mulato chili (green) 175, manila mango 171, cauliflower 154, turnip leaves 147, ancho chili 143, guajillo chili 135, tangerine 112, mulato chili 111, guava 89, quesa de tuna 88, spinach 82, tomato 76, quelites 68, green peas 67, papaya 65, maguey flower 59, lima 51, plums 46 and malva 45.

11. *Manganese*

During the investigation, it was noticed that the ash of cilantro was unusually green in color; later it was proven to be due to an extremely high manganese content (12.9 mg. %). In Sherman's tables ('41), the American food reported with the highest manganese content is oatmeal with only 4.9 mg. per 100 gm.

12. *General considerations*

The value of a food in supplying the needs of a population group is measured by the nutrient content in a daily serving rather than the content per 100 gm. Thus, although chili is rich in ascorbic acid, a large amount cannot be eaten daily. On the other hand, cereals which are relatively low in nutrient content become important sources because of the large quantities consumed.

Some exceptional foods have been revealed by these analyses. Malva is an uncultivated plant that grows abundantly on the Mexican plateau. It is cooked in much the same manner as spinach which it resembles in taste, though malva is more fibrous. It is interesting that an ordinary portion (100 gm.) of malva contains approximately 40% of the calcium, 90% of the iron, 140% of the vitamin A (as carotene) and 60% of the ascorbic acid allowances for an adult man proposed by the National Research Council. The variations of the analyses of the several samples of malva indicate that more nutritive strains can be expected through breeding and cultivation.

Charal is an inexpensive air-dried fresh water fish, usually 3 cm. to 6 cm. in length, the entire carcass of which is customarily eaten. Charales are especially rich in protein and calcium for a 30-gm. portion supplies approximately 27% of the protein and 155% of the calcium allowances of adult man.

Quesa de tuna is prepared from the fruit of the prickly pear cactus and resembles cheese in texture. Its nutritive value lies especially in its iron and ascorbic acid content. Gusanos are grubs gathered from the roots of the maguey cactus and served as a fried delicacy. They are low in nutritive value.

Pulque is a beverage prepared by the fermentation of the juice of the maguey cactus. In arid areas of the Mexican plateau, it is consumed in liberal amounts, partly to supply water. It is an inexpensive sour drink which in daily portions of 500 ml. furnishes significant quantities of minerals and vitamins, especially ascorbic acid. The ascorbic acid analysis has been confirmed by bioassay using guinea pigs.

The results indicate that sesame seed is a food rich in calcium, iron, thiamine, and niacin; guaje seed is rich in calcium, iron, carotene and thiamine; charales in protein, calcium, iron, vitamin A and niacin; parota in protein, thiamine and niacin; peanuts in protein and niacin; and calabaza seed in protein, iron and niacin. In the quantities consumed, pulque is a good source of thiamine and ascorbic acid. Piñón contains an abundance of protein and niacin, and many of the beans are rich in protein, iron, calcium and niacin.

The exceptional amounts of calcium, iron, carotene, thiamine and protein found in these Mexican foods suggest that it may be possible to nourish the Mexican people without the use of dairy and meat products. These results indicate that the food pattern of Mexico is quite different from that of the United States. Thus it would be inadvisable to base the Mexican nutrition program upon that of the United States. Instead, this program should be developed upon the nutrient composition of Mexican foods. Finally, the data of this analysis of more than 100 foods indicate that the Mexican dietary may be more adequate in ascorbic acid, phosphorus, calcium and thiamine than in riboflavin, niacin and quality-protein.

CONCLUSIONS

1. The results of the analysis of 112 samples of Mexican foods for carotene, thiamine, riboflavin, niacin, ascorbic acid, calcium, phosphorus, iron, nitrogen, ash and total solids content are presented.
2. Malva, sesame seed, charales, epazote, parota, peanut, guaje seed and calabaza seed were found to be especially nutritious foods. Many Mexican foods are richer in specific nutrients than foods in the United States.
3. The kinds and nutritional qualities of the foods of Mexico are so different from those of the United States that the nutrition program in Mexico should be based upon the nutrient content of Mexican foods.
4. It is probable that the Mexican diet is more adequate in ascorbic acid, thiamine, phosphorus, and calcium content than in riboflavin, niacin and quality-protein content.

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A STUDY OF NICOTINIC ACID RESTRICTION IN MAN [1]

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ONE FIGURE

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With the observation by Perlzweig, Levy and Sarett ('40) that the chief end product of nicotinic acid metabolism is to be found in the trigonelline fraction, and with the development of a direct method for the determination of trigonelline by Kodicek and Wang ('41), our interest was directed to the study of nicotinic acid metabolism in man with dietary restriction.

Several subjects volunteered to restrict their eating to a pellagra producing diet, low in trigonelline as well as nicotinic acid, with hopes of ridding themselves of some real or fancied ailment. Two of these adhered to the diet for a considerable period; it is with the results on these two subjects that the present report is particularly concerned.

METHODS AND MATERIAL

The subjects

Etta R. was a colored female, age 58, with complaints of stomach trouble and weakness. On a previous admission in 1930 she had pellagra. In 1936 she was operated on for removal of a uterine fibroid.

Examination revealed nothing of interest except possible cheilosis at one angle of the mouth and some pigmentation over the elbows. The tongue appeared somewhat atrophic but not red.

She adhered to the diet for 42 weeks. Throughout the greater part of the study she seemed to be contented; probably the diet and social life on the ward compared favorably with that at her home.

Chas. G. was a vagrant white male, a chronic alcoholic. He had been admitted several times previously with pellagra, and it had been customary with these admissions to put him on a pellagra producing diet for a variable period of study, so that he had come to look upon the diet as part of the cure.

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[1] The expense of this study was supported in part by a grant-in-aid from the John and Mary R. Markle Foundation.

At the present admission he was mildly intoxicated and rather badly disoriented but he was able to indicate that he wanted the usual treatment. The skin over his neck, hands and wrists showed what might have been the result of sunburn ± pellagra — very red, rather rough but without vesicles. The tongue looked fairly good. Because of his mental state and the appearance of his skin and his past history, he was suspected of being in a state of nicotinic acid deficiency. He was immediately placed on the basal diet[2] made up of common foods. The nicotinic acid content of a duplicate of this diet was found to be 2.42 mg. by biological assay.

Supplementary foodstuffs were found to have nicotinic acid, milligram per 100 gm., as follows: squash 0.44 and 0.52; white potatoes 0.73; spaghetti 1.2; onions 0.19; cabbage 0.45. The following supplements were added to the diet of Etta R.: none during the first 18 weeks and none the last 2 weeks; 50 gm. cabbage and 50 gm. onions from the eighteenth through the thirtieth week; 50 gm. cabbage and 50 gm. squash during the thirty-first and thirty-second weeks; 50 gm. cabbage and 50 gm. white potatoes from the thirty-second to the fortieth week. A supplement of 50 gm. of spaghetti with 5 gm. of cheese was included in the diet of Chas. G. throughout his study. It was assumed that the 5 gm. cheese contained no significant quantities of nicotinic acid bodies.

Chemical technique

Twenty-four hour urines were collected in gallon bottles containing 25 ml. of 5 N HCl as preservative. Each week four of the daily specimens were analyzed for creatinine, trigonelline and nicotinic acid (nicotinic acid plus easily hydrolysable nicotinic acid derivatives). All determinations were made by colorimetric methods, employing the Evelyn instrument.

Creatinine was determined by an adaptation of the usual Folin and Wu method.

Nicotinic acid was determined by a technique involving the following steps: hydrolysis with N NaOH by immersion for 45 minutes in boiling water; acidification and adsorption on Lloyd's reagent; elution with 0.5 N NaOH; neutralization and clearing of the eluate with 10% $ZnSO_4$; and finally, color development with CNBr and Elon in the presence of acid phosphate buffer.

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[2] The composition of the basal diet was: ground lean beef 25 gm., flour (not enriched) 120 gm., hominy 50 gm., oatmeal 25 gm., cream 20 gm., lard 25 gm., Nucoa 40 gm., simple syrup 150 gm., sugar as requested, synthetic fruit juice 3 servings, hard candy as requested.

Trigonelline was determined by the method of Kodicek and Wang ('41) with a few minor alterations.

Determinations were also made of the urinary output of the fluorescent substance F_2, by the method of Najjar ('44).

CLINICAL OBSERVATIONS

With a view towards increasing the nicotinic acid requirements, and relative deficiency, of the subject Etta R., 10 units of insulin were administered three times daily during the first 18 weeks. Following this she was given ultraviolet irradiation daily during the thirty-first to thirty-sixth weeks (cold quartz to entire body, 2 minutes each to anterior and posterior surfaces at 32-inch distance). Then she was exposed to x-rays during the thirty-ninth week, 200 R over the abdomen, alternating daily from anterior to posterior surfaces, and after 7 days of this two additional treatments of 150 R, the dosage being reduced because of nausea.

In spite of the restriction of nicotinic acid, irradiation, etc., nothing developed which could be called pellagra. There was some marginal redness of the tongue and some slight increase in the pigmentation over the elbows; we would have considered her condition, from the clinical standpoint, as mild nicotinic acid deficiency.

We were particularly surprised that so little effect appeared to result from the irradiation. In other patients irradiation had produced lesions which were very definitely those of pellagra, in one instance fulminating pellagra. Bean, Spies and Vilter ('44) have recently reported the appearance of the pellagra syndrome associated with therapeutic irradiation.

The skin lesions of Chas G., with removal of the sunburn factor, faded considerably during the first few days of hospitalization, but a residual roughness and redness remained which was unchanged during the 9 weeks on the basal diet. With administration of nicotinic amide there was a rather prompt improvement, the skin lesions becoming softer and less pigmented.

METABOLIC OBSERVATIONS

Cooperation in collection of specimens was excellent, judging from excretion of creatinine as an index of completeness: Etta R. excreted regularly about 1.0 gm. and Chas. G. about 1.4 gm. Since the four specimens used each week for analysis were usually from successive days, inequalities of collection periods were largely cancelled.

The average values for nicotinic acid and for trigonelline, on the four specimens analyzed, were taken as the daily excretions for the week. Weekly fluctuations, so calculated, are shown in the accompanying chart.

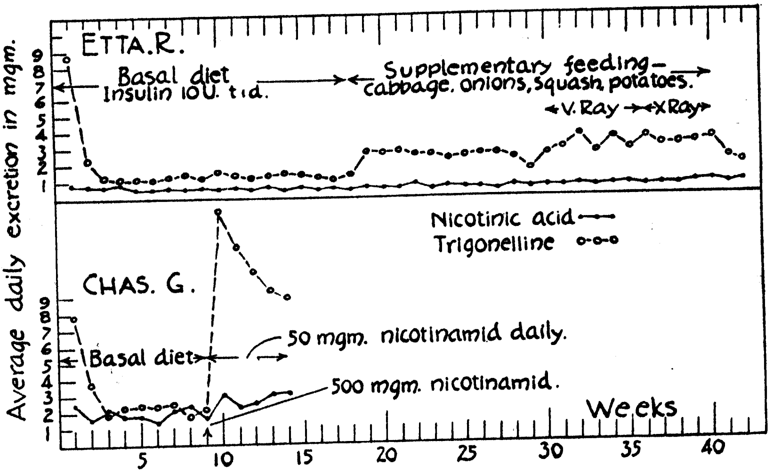

Fig. 1 Showing the average daily values for urinary excretion of nicotinic acid and trigonelline, during a study of nicotinic acid restriction.

The nicotinic acid output

The urinary excretion of Etta R. amounted to a little less than a milligram nicotinic acid daily at the start of her study, and it remained at this level to the end. This is just below the range of 1 to 3 mg. usually observed on normal controls, and might possibly be taken as an expression of her poor nutritional state. However, Chas. G., who we have reason to believe was in a somewhat more depleted state of nicotinic acid nutrition at entrance, excreted about 2 mg. daily with essentially the same intake. Also, following the test dose of 500 mg. nicotinic amide and the therapeutic administration of 50 mg. nicotinic amide daily, over a period of 5 weeks, his output was only increased to a level of about 3 mg. daily. These observations fail to show anything of value in the urinary assay of nicotinic acid as an index of the nutritional status; they are in agreement with the results of other studies in this respect (Briggs, '41; Field et al., '41; Goldsmith, '42).

The output of trigonelline

It is obvious that we have been successful in devising a diet low in trigonelline as well as nicotinic acid. The excretion of trigonelline by

Etta R. dropped within 3 weeks to about 1 mg. per day, and remained at this low level until the supplementary feedings were started; the increased output during the remainder of her study was due to preformed trigonelline in the supplements — biological assay revealed only small quantities of nicotinic acid in these foodstuffs. Chas. G. excreted a little more than 2 mg. per day during his period of restriction. Since his diet only differed from the basal diet by the supplementary 50 gm. of spaghetti plus 5 gm. of cheese, it is possible that his slightly greater output of trigonelline, was an expression of greater total metabolism.

Sarett ('42) observed, with dogs on a black tongue producing diet, that the output of trigonelline dropped progressively to zero during the course of a few months. Our failure to observe a drop below the level of the third week is no doubt due, in part, to greater quantities of nicotinic acid and trigonelline in our diets; but we also believe that the direct technique employed by us is better suited to the detection of small quantities of trigonelline than the indirect technique of Sarett.

Niacin tolerance tests

Each of the subjects, at the close of their period of dietary restriction, was given the tolerance test suggested by Sarett, Huff and Perlzweig ('42): 500 mg. of nicotinic amide was administered by mouth and the quantity of extra trigonelline plus nicotinic acid bodies excreted during the next 24 hours determined by chemical analysis. According to their observations, with a state of nicotinic acid deficiency, the "extra" excretion should be less than 25 mg. (less than 5% of the test dose).

The "extra" output of Etta R. was 76 mg., a little more than 15%; the "extra" output of Chas. G. was 38 mg., between 5 and 10% of the 500 mg. ingested. According to the Sarett et al. standard then, neither of these subjects could be called deficient. We have the feeling that this standard must indicate an advanced state of deficiency. As a matter of fact, only one of their control subjects excreted less than 17% "extra" trigonelline plus nicotinic acid; and our own observations on three subjects who only adhered to the basal diet for a short time gave the following results for "extra" output: Bryant 18.2%; Caldwell 18.3%; Higgs 14.4%. Probably, with our technique, anything less than 75 mg. "extra", or 15%, should be considered as representing deficiency. Goldsmith ('44) has recently observed that normal controls excreted more than 20 mg. "extra" within 6 hours after ingestion of only 300 mg. nicotinic amide. These results seem to be in line with our own.

The urinary assays of F_2

From the work of Huff and Perlzweig ('43) and Najjar, White and Scott ('44) it seems probable that the fluorescent substance F_2 is a methylated derivative of nicotinic acid, presumably a precursor of endogenous trigonelline.

Since F_2 has been found absent from the urine in pellagra (Najjar and Holt, '41) it has been suggested that the nutritional state with respect to nicotinic acid might be revealed by the urinary assay of F_2. From our own limited experience this seems to be true (Singal, Hall and Sydenstricker, '44); however, the value of the test has been questioned by Mickelsen ('44).

The results of the F_2 determinations are not shown on the chart for the reason that all values were zero for each of these subjects, during the early as well as the late stages of the period of restriction. F_2 did, however, appear promptly following the administration of the test doses of nicotinic amide.

It is evident, therefore, that urinary F_2 may be absent in mild states of niacin deficiency: each of these subjects had questionable skin lesions; neither subject appeared to be in a severe state of deficiency from clinical examination nor from the niacin tolerance test.

Nicotinic acid requirements

The evidence of dietary surveys suggests that the minimal niacin requirement is considerably less than the figure of 18 mg. daily which was recommended by the National Research Council. Dann ('44) has pointed out that dietary calculations from these surveys in various localities, in which neither pellagra nor gross evidence of milder deficiency were observed, indicate a nicotinic acid intake frequently as low as 5 mg. daily or less.

By analysis of duplicate diets, Winters and Leslie ('43) have found that self chosen diets of a low income group in Austin, Texas, provided a daily intake of nicotinic acid ranging from 2.7 to 9.8 mg. with a mean of 4.2 mg. The only evidence of niacin deficiency observed was some lateral redness of the tongue and red, swollen papillae.

It becomes less surprising, therefore, that Etta R., with a daily intake of about 3 mg. niacin daily over a period of 42 weeks, exhibited only lateral redness of the tongue and some increase in the pigmentation over her elbows. However, the appearance of minimal lesions in this subject, and the fact that the lesions of Chas. G. failed to heal on an intake of about 3 mg., suggests that we were working with intakes somewhere near the minimal human requirement, probably a little below the minium requirement for the conditions of our study.

One factor which must be considered is the possibility of intestinal biosynthesis of niacin. Najjar and Holt ('43) have observed evidence of biosynthesis of thiamine; and evidence from the same laboratory (Najjar, Johns, Mediary, Fleishman and Holt '44) has just appeared, which suggests that biosynthesis of riboflavin may be sufficient for human requirements. So, an analogous intestinal biosynthesis of niacin might provide a protective supplement and complicate a metabolic study such as the present one. We have a single observation bearing on this point: Etta R. took 4 gm. of sulfaguanidine daily, during her twenty-ninth week and, as may be observed on the chart, there was a slight but definite drop in the output of trigonelline.

Each of these subjects had been treated previously, for classical pellagra. Consequently, the lack of an individual idiosyncrasy, if such a factor really exists, cannot be invoked to explain their failure to exhibit the syndrome during this study.

Except for the 50 gm. of hominy, our basal diet contained no corn. Because of the association of maize eating and clinical pellagra it has been suggested by Chick ('33) that a toxic substance in corn tends to neutralize the pellagra preventive vitamin. Possibly we have observed all which should have been expected on a corn poor diet. It is conceivable, of course, that something in corn might inhibit the intestinal biosynthesis of nicotinic acid.

SUMMARY

Two subjects, each of whom previously had been a pellagra patient, restricted themselves to a diet low in trigonelline and providing only about 3 mg. of nicotinic acid daily, one for a period of 9 weeks and the other for 42 weeks.

Each had minimal lesions of nicotinic acid deficiency at the start, but in neither was there any significant development in the direction of pellagra.

The nicotinic acid excretion of one remained low throughout the study; the other remained at a normal level.

The trigonelline output in each case dropped to a low level within 3 weeks, but showed no tendency to fall to a lower level with prolonged restriction.

Niacin tolerance tests in each case were interpreted to indicate a mild state of deficiency.

Tests for the fluorescent substance F_2 were zero at the start as well as at the close of the periods of restriction.

It is suggested that the failure in the development of pellagra may have been due possibly to intestinal biosynthesis of nicotinic acid, and possibly to the fact that the diet contained little corn.

Apparently, under the conditions of our study, the 3 mg. daily of the diet provided an intake somewhere near the minimal niacin requirement.

ACKNOWLEDGMENT

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THE EFFECT OF THE LEVEL OF PROTEIN IN THE DIET ON THE UTILIZATION OF VITAMIN A[1]

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This is a report upon the third phase of an experiment planned to determine the effect of various dietary factors upon the utilization of vitamin A. In the two previously published studies, it was reported by Muelder and Kelly ('41, '42) that the caloric intake was responsible for greater gains in weight than was the increased unitage of vitamin A, and that the level of fat in the diet did not significantly increase the amount of weight gained. Neither the caloric intake nor fat level affected the number of "abscesses". In the present study, the effect of the level of protein in the diet upon the utilization of vitamin A is considered.

Other investigations have indicated that the utilization of vitamin A might be affected by the level of protein although there is no work with which this study is precisely comparable. Randoin and Queuille ('34) found no effect upon the time of development of xerophthalmia in young rats on a vitamin A deficient diet with varying proportions of protein; however, cessation of growth was delayed in rats receiving 27% and 37% protein. Sampson et al. ('32) reported no appreciable alteration of the absorption of nitrogen, but an increase in nitrogen metabolism and a decrease in rate of gain in body weight per unit weight of food ingested when rats were being depleted of vitamin A. Emerique ('36) obtained similar results. Basu and De ('41) found that 18% of protein in the ration considerably increase liver storage of vitamin A over that stored on a ration containing 8% protein when both groups of rats received 100 I.U. of vitamin A daily. Baumann, Foster and Moore ('42) found that with low protein diets there was less storage of vitamin A in the liver and that vitamin A previously stored in the liver was more quickly depleted.

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EXPERIMENTAL PROCEDURE

In this investigation, triads of rats of the same sex and litter were started on the vitamin A deficient diet containing an 18% level of protein (table 1) at a weight of 50 to 55 gm. and an age of 25 to 28 days. At depletion, as determined by failure to gain weight for 1 week and evidence of xerophthalmia, the basal ration of the triads of rats was changed, each member of the triad receiving an equi-caloric basal diet containing, respectively, 9, 18 and 36% protein. Each member of the triad received the same level of vitamin A supplement,[3] 0, 1, 3 or 6 I.U. Twenty-four triads, twelve male and twelve female, were placed on each level of vitamin supplement making a total of 288 animals used in the experiment. The methods of preparation and of feeding the supplements were the same as used previously (Muelder and Kelly, '41). The animals were weighed weekly and the food consumption was checked daily. The food consumption of a triad was restricted to the level of the poorest feeder in that triad.

TABLE 1

Composition of basal rations varied with respect to source of protein.[1]

INGREDIENTS	DIET DURING DEPLETION	EXPERIMENTAL DIETS		
	%	%	%	%
Casein, vitamin A-extracted + 0.05% cystine	18	9	18	36
Cornstarch, irradiated	73	82	73	55
Fat, refined cottonseed oil	5	5	5	5
Salt mixture, Osborne and Mendel	4	4	4	4

[1] Throughout the entire experiment 0.5 gm. bakers' yeast was fed daily as a supplement to supply the B vitamins.

Criteria used to measure the response of the animals to vitamin A were: growth, as shown by gain in weight and by length of rat, and numerical total of the incidence of accumulations of keratinized epithelial cells, called "abscesses". Differential leucocyte counts were made by standard procedure from blood smears of a number of animals from each group at the end of the experimental period. Measurements of the total thicknesses of the midlingual and midlabial dentin were also made as an index of the utilization of vitamin A. After death, the animals were decapitated and the heads fixed in a 4% aqueous solution of formaldehyde. Transverse sections of lower left and right incisors were prepared by grinding on medium and fine carborundum stones. With a filarmicrometer eyepiece, calibrated to a stage micrometer, measurements were recorded for the narrowest widths of labial and lingual dentin within the area defined by the width of the pulp cavity and parallel to the lines of dentin tubules.

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[3] Reference cod liver oil (U. S. Pharmacopeia, XI).

RESULTS AND DISCUSSION

The animals were depleted of vitamin A within an average of 6.3 weeks, the growth plateau occurring at an average weight of 116.2 gm. The food intake averaged 49.9 gm. per week during the depletion period.

Growth responses and food utilization

A summary of the data, expressed as combined averages for the different levels of protein fed, for the food intakes and weight changes, together with the length of rats and number of "abscesses" evident at autopsy is given in table 2. Statistical analyses of the means were made using the formula "t" = $\frac{M - M_1}{\frac{(\sigma)^2}{n} \quad \frac{(\sigma_1)^2}{n_1}}$. From these analyses, it was found that the level of protein had no very significant effect upon gains in weight on any level of vitamin A. A slightly significant gain in

TABLE 2

Food intake, weight changes, and autopsy findings expressed as combined averages for males and females.

NO. OF ANIMALS	LEVEL OF VITAMIN A PER DAY	LEVEL OF PROTEIN	AV. WEEKLY FOOD INTAKE	AV. WEEKLY CHANGE IN WEIGHT	GM. GAIN Gm. food intake	"ABSCESSES" EVIDENCE AT AUTOPSY	LENGTH OF RAT AT AUTOPSY
	I.U.	*%*	*gm.*	*gm.*		*number*	*inches*
24	0	9	23.93 ± .97[1]	− 9.61 ± .85		4.88 ± .25	12.98[2]
24		18	22.97 ± .96	− 8.11 ± .84		5.08 ± .27	12.93
24		36	23.66 ± .90	− 9.39 ± .86		4.96 ± .29	12.50
24	1	9	34.92 ± .62	+ .007 ± .43	.0002	2.21 ± .33	12.95 ± .13
24		18	34.77 ± .60	+ .96 ± .48	.0259	1.62 ± .25	12.88 ± .18
24		36	34.76 ± .60	+ .35 ± .47	.0107	1.71 ± .30	12.91 ± .14
24	3	9	44.99 ± .82	+ 4.15 ± .47	.092	1.29 ± .27	13.17 ± .17
24		18	45.05 ± .81	+ 5.80 ± .57	.129	1.38 ± .23	13.29 ± .19
24		36	45.01 ± .82	+ 5.18 ± .53	.115	1.25 ± .26	13.29 ± .18
24	6	9	41.04 ± .80	+ 3.58 ± .47	.087	1.71 ± .27	13.38 ± .12
24		18	41.00 ± .79	+ 4.74 ± .53	.116	1.58 ± .35	13.40 ± .13
24		36	41.04 ± .80	+ 3.56 ± .47	.087	1.67 ± .22	13.12 ± .18

[1] Arithmetic mean ± standard error.

[2] Too few animals were measured before death to warrant statistical analysis of data.

weight in favor of the 18% protein level over the other 9 and 36% levels was shown at each unitage of vitamin A. As might be expected, significant gains in weight were shown on all levels of protein as the unitage of vitamin A was increased up to 3 I.U. per day. These results confirm previous findings that the level of vitamin A in the diet is an important factor in promoting gains in weight, and that vitamin A is able to function to stimulate growth when combined with diets containing a wide range of protein level.

The averages in table 2 represent gains in weight of both male and female animals. In separating the results according to sex, it was found that when either 3 or 6 I.U. of vitamin A were given, the males showed significantly greater gains than the females on each level of protein. The difference between the gain in weight of the sexes agrees with the findings of Palmer and Kennedy ('31) and Coward et al. ('31). There were no significant differences favoring one level of protein for either sex.

Since accurate measurements of the length of all the rats could not be made, the data were not analyzed statistically. The few figures obtained do not show any variation in length of the animals on the various levels of protein. Rats on a higher level of vitamin A were slightly longer.

The administration of even 1 I.U. of vitamin A produced a highly significant reduction in the number of "abscesses" over those occurring in the animals receiving none. Three and 6 I.U. brought no further significant reduction, except a slight decrease in favor of 3 I.U. over 1 I.U. on the 9% level of protein. Some pathological lesions were present after 6 weeks on a diet containing as much as 6 I.U. of vitamin D daily, which agrees with the observations reported by Richards and Simpson ('34).

Use of differential leucocyte counts as a criterion for utilization of vitamin A

Some investigators have indicated that differential leucocyte counts were altered in vitamin A deficiency states. The data presented in table 3 were collected in an effort to determine whether or not the blood picture would serve as a satisfactory criterion for the utilization of vitamin A under the conditions of this experiment. The small number of blood smears made from animals receiving no vitamin A is due to the impossibility of obtaining satisfactory blood samples because of unpredictable times of death among these groups. Total leucocyte counts probably fall within the normal limits for rats in all instances, although information on normal animals is limited and varies a great deal. The few data from animals receiving no vitamin A indicated total leucocyte counts almost twice as high as those in other animals. It would appear that the protein levels of the diets used in this study had no effect on the total or differential leucocyte counts. There is, however, an indication of increased polymorphonuclear cells among the animals receiving no vitamin A and those receiving only 1 I.U. of vitamin A daily. The possible disturbance of polymorphonuclear cells which has been noticed here agrees with the findings of Abbott and Ahmann ('38) and of Turner and Loew ('31); on the other hand, Sure, Kik and Walker ('31) found no evidence of change in the differential leucocyte count in vitamin A deficiency. Since the techniques for carrying out differential leucocyte counts are standardized and easily done, it would appear that this criterion for judging utilization would warrant further study with larger numbers of animals.

TABLE 3

Summary of differential leucocyte counts.

NUMBER OF RATS FOR TOTAL COUNTS	LEVEL OF PROTEIN IN FOOD	LEVEL OF VITAMIN A IN FOOD PER DAY	TOTAL LEUCOCYTE COUNT	DIFFERENTIAL COUNTS					
				Polymorphonuclear cells	Stabs	Large lymphocytes	Small lymphocytes	Myelocytes	Monocytes
	%	I.U.	M/cmm.	%	%	%	%	%	%
3	9	0	18.2	31	6	7	56	0	0
3	18	0	16.0	16	1	7	68	1	7
2	36	0	16.0	14	15	13	49	7	2
7	9	1	7.4	23	3	11	58	5	1
7	18	1	8.1	23	3	8	62	3	1
7	36	1	7.5	12	3	12	67	4	2
8	9	3	9.6	12	5	13	66	3	1
8	18	3	7.3	15	4	10	67	3	1
8	36	3	8.0	8	4	14	69	4	1
9	9	6	9.3	12	4	12	69	2	1
10	18	6	8.5	9	5	12	70	2	2
9	36	6	11.4	9	5	8	76	2	0

Use of dentin widths in transverse ground sections of incisors of rats as a criterion for utilization of vitamin A

A study of the ratios of midlingual to midlabial dentin-widths in transverse ground sections of lower incisors of some of the experimental animals was made. Sections of incisors from rats receiving no vitamin A, and many of those from animals receiving 1 I.U. or even more vitamin A daily showed increased thickness of enamel-covered dentin, narrow cementum covered dentin, and irregular pulpal outline. All of these distortions of the normal growth pattern are characteristic of vitamin A deficiency, as described by Schour, Hoffman, and Smith ('41). The ratio of midlingual to midlabial dentin in the normal rat incisor is 1:1.

The ratios of lingual to labial dentin furnish little if any basis for determining the effect of protein on the utilization of vitamin A. Animals receiving no vitamin A or 1 or 3 I.U. of vitamin A showed dentin ratios of approximately 1:2 at all levels of protein except in one case. In the group receiving 3 I.U. of vitamin A and 18% protein, improvement in the condition of the teeth as shown by a ratio approximately 1:1 was observed. This small group of 4 animals does not justify the conclusion that the 18% level of protein allowed better utilization of vitamin A than 9% and 36% protein. It was not until 6 I.U. of vitamin A were included in the diet that ratios of dentin widths approached the normal relationship. These ratios were approximately 1:1 regardless of protein intake.

SUMMARY

The effect of 9, 18, or 36% of protein in the diet on the utilization of vitamin A was studied in 288 rats. The level of protein had little effect upon the utilization of vitamin A as judged by weight gains, length of the rat, and incidence of foci of keratinized epithelium. The level of vitamin A intake was directly related to rate of gain up to the level of 3 I.U. per day regardless of the level of protein in the diet. There were slightly greater gains in weight on the 18% level of protein regardless of the level of vitamin A than on other quantities of protein.

Increase in polymorphonuclear cells was observed on low vitamin A intakes.

The teeth did not show a normal midlingual to midlabial dentin ratio until 6 I.U. of vitamin A were present in the diet.

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THE NUTRITIVE VALUE OF THE FATTY ACIDS OF BUTTER INCLUDING THEIR EFFECT ON THE UTILIZATION OF CAROTENE[1]

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TWO FIGURES

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Most of the current studies of the comparative nutritive value of various fats have relied on the effect on growth as the sole criterion and have dealt with the whole fat rather than with its respective fatty acids. There is need for further information on the special physiological functions of specific acids.

That highly unsaturated fatty acids are essential in the diet has been established (Burr and Burr, '29; McAmis, Anderson, and Mendel, '29; Burr and Burr, '30; Evans and Lepkovsky, '32). The acids which will produce some curative effects in rats suffering from deficiency of fat in the diet are linoleic, linolenic, arachidonic, decosahexenoic and hexahydroxystearic (Burr and Barnes, '43).

The growth-promoting value of fatty acids of different degrees of saturation is still under investigation. Boutwell, Geyer, Elvehjem and Hart ('41) found that the saturated fatty acids of butter and especially the unsaturated acids which had been artificially saturated, when mixed as glycerides with corn oil and incorporated in a skimmed milk diet, produced better growth in rats than the unsaturated acids or butter fat itself. On the other hand, the work of Loosli, Lingenfelter, Thomas and Maynard ('44) suggests that unsaturated acids, as found in corn oil, may have a particular value for the growth of suckling rats.

The following is a report of a preliminary study of the nutritive value of some fractions of the fatty acids of butter. We have found that rats receiving the unsaturated acids of butter as the major source of fat in a normal diet, supplemented with raw carrot and Drisdol (a source of vitamin D), store more vitamin A in their livers than rats

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receiving the saturated or volatile acids. The diets containing the unsaturated acids also seem to be more efficient in producing growth, probably due to better absorption.

PROCEDURE

1. The preparation of fatty acids

Dry butter fat was saponified with 10% alcoholic potash by refluxing over steam for 2 to 3 hours. Most of the alcohol was removed by distillation and the remainder by suction over warm water. The soap was mashed with a little water and acidified, with cooling, by an excess of dilute sulphuric acid. Steam distillation was carried out until about 5 liters of distillate had been collected. This was extracted with ether and the extract was distilled to remove most of the ether. The concentrated solution was made to volume and titrated to determine the amount of acid as butyric.

The residue containing the non-volatile fatty acids was extracted with distilled ether. After the extract had been washed and dried, the ether was removed and the fatty acids were separated into the "solid" and "liquid" groups by use of the lead salt separation. Each group of acids was then dissolved in ether, washed, dried, freed from ether and refluxed with methyl alcohol containing 1 to 2% dry hydrogen chloride gas. The methyl esters so obtained were taken up in ether, washed with water, dilute sodium carbonate, and again with water. They were then freed from all water and ether and subjected to fractional distillation. The apparatus used was patterned after that of Longenecker ('37). Three fractions were taken off, the middle one being redistilled and added to the other two in such a way as to obtain finally two fractions of equal weight, having average molecular weights as different as possible. Each fraction was then saponified with alcoholic potash, freed from alcohol, acidified with an excess of dilute sulphuric acid and extracted with ether. The ether extracts were washed, dried, and the ether removed. Constants were determined on all fractions which were then stored under nitrogen at 0°C.

The average titration equivalents obtained for the high and low molecular weight liquid portions were 279 and 226; those for the high and low molecular weight solid portions were 271 and 248. The average iodine values for the high and low molecular weight liquid portions were 98 and 35; those for the high and low molecular weight solid portions were 7.8 and 0.6.

2. *The diet*

The diet used was that known as the Sherman B (Sherman and Campbell, '24), modified for the purposes of our experiment. Each 100 gm. contained 10 gm. of the test fat or fatty acid, 23.3 gm. of skimmed milk powder, and 66.6 gm. of whole wheat flour. Sodium chloride was added in amounts equal to 2% of the weight of the flour. This made the amount of test fat in the diet 9.9%. Small batches of the diets were prepared every few days and stored in the refrigerator. In the first two experiments the solid acids were melted and poured over the rest of the mixture, the whole mass being pulverized in a mortar. In experiment 3 they were first pulverized and then put through a sieve with the rest of the ingredients. The butter diet was made with whole butter.

An exception was made in the case of the diet containing the volatile acids. The 100 ml. of ether solution of these acids obtained from each pound of butter contained about 19 gm., calculated as butyric acid. This amount was added to 300 gm. of the basal diet in the small portions required for each day's feeding. The fat content of this diet was about 6%.

Six test preparations were used. The diets containing them will be designated butter, volatile, low liquid, high liquid, low solid, high solid. In addition to the diets the rats received 1 drop of Drisdol (crystalline vitamin D from ergosterol in propylene glycol) and 3 gm. of raw carrot per week. They were fed ad libitum.

3. *The experiments*

Three duplicate experiments were run, except that the first was continued for 3 weeks, and the next two for 5 weeks. Each experiment used twenty-four male rats, put on the diets at 21 days or after, and distributed by litter and weight as fairly as possible. They were kept in individual cages. The average starting weights in the three experiments ranged from 44.0 to 46.1, 45.1 to 46.1 and 34.8 to 35.0 gm.

The rats were weighed weekly and their food consumption per week was determined. In experiment 3 the feces were collected each day during the final 2 weeks on the diets and stored in the refrigerator.

The rats were killed by illuminating gas. Autopsies were performed for the purpose of noticing any gross abnormalities. The livers were saved for analyses of vitamin A and many other organs were weighed.

4. *Methods of analysis*

a. Vitamin A. The method used for extraction of vitamin A from the liver has been described elsewhere (McCoord and Luce-Clausen, '34). The vitamin A content of the petroleum ether extracts was determined by means of the Evelyn photoelectric colorimeter (Clausen et al., '42). The results are expressed in terms of International Units (I.U.), one of our blue units being equal to 3.8 I.U.

b. Lipids of the liver. A composite of half the liver tissue of each rat in a group, from experiment 3, was used for the analysis. This was enough for single determinations only. The tissue was refluxed over steam with 5% aqueous sodium hydroxide, and acidified with dilute sulphuric acid. The extraction was made first with ether and then, after removing the ether, with petroleum ether. One volume of the petroleum ether extract was shaken in a separator with two volumes of 50% alcohol containing potassium hydroxide. This made it possible to separate the fatty acids as soap from the unsaponifiable material in the petroleum ether layer. The latter was drawn off into a small suction flask, the petroleum ether sucked off, and the flask weighed. The other portion was heated on the steam bath to remove most of the alcohol and was then acidified with dilute sulphuric acid. Extraction was done with petroleum ether, the extract washed with water, and the petroleum ether removed by use of the small suction flask which was then weighed.

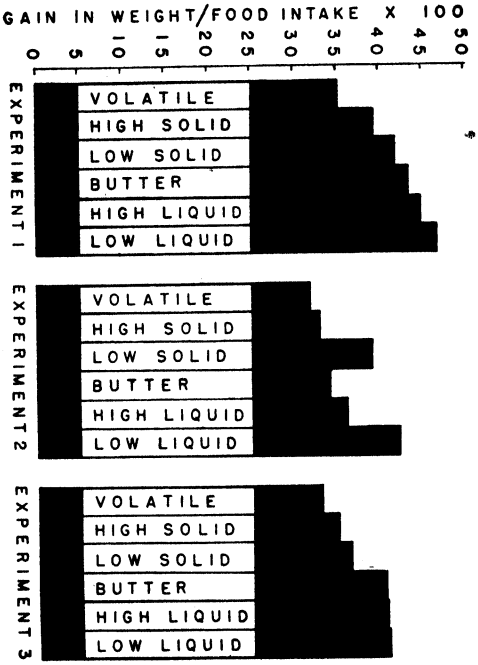
c. Lipids of the feces. Two 5-gm. samples of the feces from each of three rats in a group (experiment 3 only) were pulverized and refluxed on the steam bath with 10% alcoholic caustic soda. They were diluted with 3 parts of water, containing an excess of sulphuric acid, and extracted, first with ether, and then after the removal of ether, with petroleum ether. The latter extract was washed with water and the petroleum ether layer was taken off into a small suction flask. The solvent was removed and the flask weighed. In most cases the lipid material from the duplicate samples was combined before being used for the determination of iodine number.

d. Constants. Iodine number was determined by the method of Wijs. The usual saponification method was followed whether the material to be tested was a fat or a fatty acid, but in the latter case we use the term "titration equivalent" instead of "saponification equivalent".

RESULTS

1. *Growth*

A study of the gains in weight made in relation to the amounts of food eaten suggests a superiority for the unsaturated acids. This relation was studied by determining the per cent efficiency of the diet in the following manner. The ratio of the gain in weight over the food consumption for each rat while on the diet was determined and multiplied by 100. The average of the values so obtained for the four rats of a group is reported as the efficiency of that diet in an experiment. As shown in figure 1 the efficiency values for the low liquid diet, in all three experiments, are at the top. They are 46.8%, 42.3% and 41.0%, respectively. The corresponding values for the high liquid diets are 44.9%, 36.2% and 40.7% which means that in the first two experiments the high liquid diets were inferior to the low liquid ones. Also in those experiments the values for butter, 43.4% and 34.2%, are still lower while in the third experiment the value of 40.6% is practically identical with those of the liquid diets. With the exception of the low solid group of experiment 2 with a value of 39.2% there is a progressive decrease in values from butter to low solid to high solid to volatile. The repetition of this order throughout three experiments probably means that there

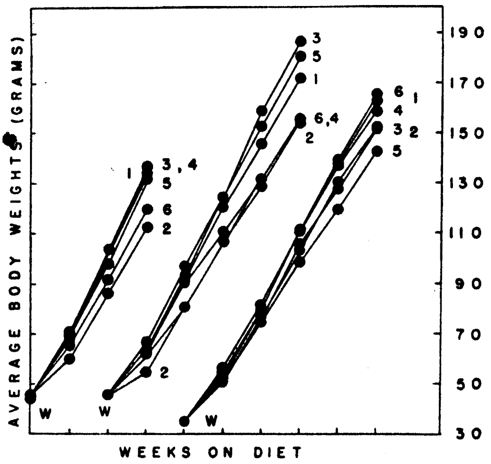


Efficiency of diets

Fig. 1 The efficiency of the diet is determined by dividing the total gain in weight of each rat while on the test diet by the total amount of food eaten and multiplying by 100. Each column represents the average values for four male rats.

is a real superiority of the liquid and butter diets over the solid and volatile diets, with the possible exception of the low solid diet. These results are probably not related to the amount of vitamin A available inasmuch as the rats receiving the butter diet were getting much more of the vitamin than any of the others and yet their growth was not outstanding.

The growth curves, figure 2, drawn without any reference to the amounts of food eaten do not give clear evidence for the superiority of the unsaturated fatty acids. Evidently this might have been demonstrated by such curves if the food intake of the rats had been standardized, instead of being unrestricted. The butter groups were among the best growing in each experiment and the volatile acids groups were among the poorest growing. Any other conclusions would require proving through the use of large numbers of animals.



Growth curves

Fig. 2 These curves represent the results for experiments 1, 2, and 3, respectively. Body weights are plotted against the number of weeks on the test diets. Each spot is the average weight of four male rats. W indicates the average weaning weight. The abscissa is marked off in intervals, each indicating 1 week. The curves for the different diets are numbered to indicate: 1 — butter diet; 2 — volatile acid diet; 3 — low liquid diet; 4 — high liquid diet; 5 — low solid diet; 6 — high solid diet.

2. *Storage of vitamin A in the liver*

The diets containing the liquid fatty acids appear to have been superior also in helping the rats to store vitamin A in their livers, when they were being fed raw carrot as its source. In table 1 the total amounts of vitamin A stored in the livers are recorded, as averages for the four rats in the group. The values for the animals receiving butter are very high compared to the rest, as would be expected from the fact that they were getting an added source of the vitamin in the butter. Of the groups which had the 3-gm. raw carrot per week as its source, those on the liquid diets stored the most vitamin A. The average values for the six groups on the liquid diets were higher than any of the average values for the six groups on the solid acid diets. There was overlapping, however, of individual results. The amounts of vitamin A stored in the livers of the groups receiving the volatile acids were very low, probably in part because the rats tended to have diarrhea.

TABLE 1

Vitamin A in the livers of rats fed the diets specified.

Each figure is the average value in international units for 4 male rats. Concentration is expressed as units vitamin A per 100 gm. wet tissue. Standard deviations are included.

	EXPERIMENT I		EXPERIMENT II		EXPERIMENT III	
	Total	Concentration	Total	Concentration	Total	Concentration
Butter	444.7 ± 62.5	7540 ± 816	457.2 ± 72.5	6678 ± 1459	377.3 ± 29.8	5203 ± 506
Volatile	30.9 ± 12.4	700 ± 366	44.1 ± 7.0	718 ± 122	32.3 ± 6.1	477 ± 102
Low liquid	141.1 ± 41.3	2235 ± 537	125.5 ± 34.2	1415 ± 420	135.5 ± 64.2	1966 ± 1102
High liquid	139.4 ± 45.4	2206 ± 716	133.2 ± 53.3	2119 ± 1179	152.7 ± 44.0	1893 ± 505
Low solid	66.0 ± 9.8	1247 ± 91	71.1 ± 27.9	1027 ± 433	86.7 ± 26.8	1193 ± 196
High solid	75.8 ± 23.2	1585 ± 621	68.4 ± 19.4	1207 ± 660	88.0 ± 22.9	1161 ± 305

Almost the same conclusions can be drawn from a study of the concentration of vitamin A in the livers, also reported in table 1. The values for the butter groups are very high and for the volatile acids groups are very low. With one exception the average values for the groups on the liquid acid diets are higher than those for the solid acid diets.

For statistical confirmation of this conclusion that there was a real difference in the total amount of storage of vitamin A in the livers, depending upon whether the diet contained the liquid or the solid acids, we made comparisons of homogeneity between various groups. As the Snedecor F values for the three low liquid groups lay within the 5% point these groups were combined. Similarly, combinations of the low solid, of the high liquid, and of the high solid groups proved to be justified. The same result was obtained when we considered the combination of the low liquid with the high liquid groups, and of the low solid with the high solid ones. However, a Snedecor F value of 30 (1%

point is 7.2) found for a combination of all 48 vitamin A values satisfied us that there is a real difference in the amount of storage between the liquid and the solid groups.

3. *Lipids of the liver*

No gross difference in the lipid composition of the liver from the different groups was observed. As seen in table 2 the values for the petroleum ether soluble unsaponifiable material were fairly constant. Since only one determination was made on a composite sample from each group no significance can be attached to the exception found in the case of the high liquid group.

TABLE 2

Lipid composition of livers of rats on following diets.

	BUTTER	VOLATILE	LOW LIQUID	HIGH LIQUID	LOW SOLID	HIGH SOLID
Unsaponifiable (gm./liver)	0.024	0.026	0.024	0.047	0.023	0.029
Total fatty acids (gm./liver)	0.16	0.21	0.21	0.17	0.19	0.18
Iodine value of fatty acids	128.6	113.7	108.8	106.9	105.6	115.2

Neither was there any great variation in the total amount of fatty acids per liver. However, the iodine value of the fatty acids of the livers from the butter group was 128.6 which is definitely higher than that of any of the other groups whose iodine numbers ranged from 105.6 to 115.2

4. *Lipids of the feces*

In the final column of table 3 we have listed, under the heading of per cent absorption the percentage of the test material ingested which was not recovered in the feces. We assume that the portion not recovered in the feces was absorbed but it is not necessarily true that the portion recovered in the feces represents unabsorbed material. In fact, in the butter, volatile, low liquid, and high liquid groups the fatty acids fed were probably completely absorbed, the fat obtained in the feces being excretory. We base this conviction on the facts that only small amounts of fecal fatty acids were obtained, and that the values were similar for the different diets, as were the iodine numbers. For these groups the percentage absorption ranged between 89 and 97, and the iodine values between 52.4 and 79.0.

In the low solid group the average percentage absorption for the three animals studied is 71.3, that of the high solid groups 42.2. These acids were not so well absorbed as the liquid acids and there is a difference in absorption between the low and high solid groups, probably due to an increased concentration of long chain acids in the latter. The molecular weights of the low and high solid acids fed averaged about 248 and 271, those of the fatty acids obtained in the feces averaged 261 and 282. It appears then that either selective absorption or selective excretion occurred, with a tendency to get rid of the longer chain acids. The melting points, on the other hand, of the mixtures fed and of those obtained did not vary much from 55°C. The average iodine value of the low solid acids increased from 0.6 for those fed to 15.4 for those obtained from the feces. That of the high solid acids fed was 7.8 and for the fatty acids obtained 13.5.

TABLE 3

Absorption of fat during final 2 weeks on the diets.

DIET	RAT NO.	DRY WEIGHT FECES	FATTY ACID	TOTAL FATTY ACID EXCRETED	IODINE VALUE	ABSORPTION
		gm.	%	*gm.*		%
Butter	49	12.2	5.6	0.68	52.4	95.2
Butter	51	11.7	6.6	0.77	53.7	93.2
Butter	52	14.1 Av.=12.7	7.0 Av.=6.4	0.99 Av.=0.81	67.4	90.7 Av.=93.0
Volatile	53[1]	12.0	5.2	0.62	65.4	92.0
Volatile	55[1]	13.9 Av.=12.9	6.3 Av.=5.7	0.87 Av.=0.75	70.5	89.2 Av.=90.6
Low liquid	57	14.2	5.3	0.75	71.5	95.4
Low liquid	58	10.1	4.4	0.44	55.1	96.6
Low liquid	60	10.4 Av.=11.5	7.9 Av.=5.9	0.82 Av.=0.67	64.1	93.1 Av.=95.0
High liquid	61	13.5	6.7	0.91	77.3	93.0
High liquid	62	14.4	6.7	0.97	79.0	93.0
High liquid	64	13.8 Av.=13.9	6.6 Av.=6.7	0.91 Av.=0.93	73.4	93.8 Av.=93.3
Low solid	65	17.4	19.0	3.31	14.9	77.9
Low solid	66	25.3	28.4	7.18	12.8	55.2
Low solid	67	11.4 Av.=18.0	18.4 Av.=21.9	2.10 Av.=4.20	18.5	80.8 Av.=71.3
High solid	69	24.4	35.4	8.65	13.5	43.4
High solid	70	25.4	36.1	9.17	13.9	46.7
High solid	71	28.1 Av.=26.0	39.4 Av.=37.0	11.09 Av.=9.64	12.5	36.5 Av.=42.2

[1] Rats were not having diarrhea during this time.

DISCUSSION

The greater efficiency of the unsaturated acids in producing gains in weight has been reported by other workers. In 1927 Ozaki studied the comparative gains in weight made by rats being fed various fatty acids as 10% of the diet and reported the following order: oleic 39.0 gm., palmitic 26.0 gm., stearic 12.5 gm., myristic 6.0 gm., and lauric 0.5 gm. Loosli et al. ('44) studied the growth of suckling rats as influenced by feeding to the mothers at parturition an ether-extracted purified diet supplemented with test fats. No increase in the weight of the young could be produced by adding ethyl linolate to the basal diet, nor by adding 10% hydrogenated corn oil or hydrogenated coconut oil, even though the latter was known to have been well absorbed. But the use of 10% corn oil, which is a good source of oleic acid as well as of linoleic, caused an increase in the average weight of the litters at 17 days from 110.6 to 129.1 gm. There thus appeared to be an advantage of unsaturated fatty acids, other than that of supplying linoleic acid. We assume that our diets, inasmuch as they contained two-thirds whole wheat flour, were adequate with respect to this acid, and yet those containing the liquid acids, particularly of lower molecular weight, tended to be more efficient in causing gains in weight than those containing the solid acids.

Much of the difference in efficiency between the liquid and solid acid diets can be explained by the poorer absorption of the solid acids. For instance, assuming 90% absorption of the non-fat solids and a constancy of the values of table 3 for fat absorption, and recalculating the efficiency by the formula $\frac{\text{gain in weight}}{\text{calories of absorbed food}} \times 100$, the following results were obtained for experiment 3: low liquid 10.1, high liquid 10.3, low solid 9.4 and high solid 9.7.

Also, we found an improved utilization of the carotene from carrot in the rats receiving the unsaturated acids. This does not seem to have depended on the molecular weights of the acids as the high and low groups produced the same results. Nor does the fact that there was a wide divergence in the degree of unsaturation of the two groups of unsaturated acids seem to have made any difference. We must conclude then that the effective acid or acids occurred in both liquid acid diets in adequate amounts. Since corn oil is a particularly good source of unsaturated acids it may be that its value in the experiments of Loosli et al. ('44) consisted in making more available the carotene fed in hydrogenated coconut oil. They do not report observations of the vitamin A content of the livers of the young.

The possibility of vitamin A or carotene of the butter having been carried over with the fractions of unsaturated acids has been considered. It does not seem possible, however, that the vitamin could withstand the chemical processes used or the distillation at 3 mm. pressure. Tests on the preparations with antimony trichloride gave either a zero or very slight reading in the Evelyn photoelectric colorimeter which did not differ between the liquid and the solid fractions.

The smaller storage of vitamin A in the livers of our rats fed the diets containing the solid acids might be attributed entirely to poorer absorption of these acids than of the liquid acids. Some of the carotene might have been dissolved in the unabsorbed acids and carried out with them as fat soluble vitamins are known to be carried out by mineral oil. If that were true, however, we should expect the amount of absorption of the vitamin to parallel the amount of absorption of the fat, and this is not the case. The amounts of vitamin A stored in the livers of the rats ingesting the low solid acids were practically identical with those of the rats fed the high solid acids and yet there was a difference in the amount of absorption of 29%, the low solids being 71.3% absorbed and the high solids 42.2%.

The question of how much fat in the diet is necessary for optimal utilization of the carotene from carrot has not been settled, nor do we know how much the nature of the fat influences the degree of absorption. It would appear that the food absorbed from the high solid diet had a fat content of at least 5%. This is calculated on the basis of a fat content of the flour of 1.9% and an absorption of the high solid acids of 42.2%. A much greater amount of fat was absorbed from the low solid diet.

CONCLUSIONS

When the fatty acids of butter were obtained in five fractions designated as volatile, low molecular weight liquid, high molecular weight liquid, low molecular weight solid and high molecular weight solid, and substituted for the milk fat in a normal diet, the following results were observed:

1. The efficiency of the butter diet in producing gains in body weight was either matched or improved upon by the liquid acid diets. With the exception of one group out of six the solid acid diets were inferior to the butter diet in this respect, due largely in all probability to poorer absorption of the solid acids. The diet containing the volatile acids was the poorest. When the results on growth were studied without reference to the amounts of food eaten, then the liquid fractions did not show a consistent superiority over the solid ones.
2. The liquid acids were absorbed very well but the average absorption of the low solid acids was 71.3%, and of the high solid acids only 42.2%.
3. The lipid composition of the livers studied showed no real differences.

4. The nature of the fat being absorbed seemed to be of importance in the utilization of carotene from carrot by the rat. The amount of storage of vitamin A in the livers of the rats fed the liquid acid diets was greater than that in the livers of the rats fed the solid acid diets. In the six liquid groups the average amounts of vitamin A stored varied from 125.5 to 152.7 I.U. Corresponding values for the six solid groups ranged from 66.0 to 88.0 I.U.

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THE EFFECT OF ATABRINE ON THIAMINE DEFICIENCY IN THE YOUNG RAT[1]

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ONE FIGURE

(Received for publication January 31, 1945)

Previous studies on the relation of nutrition to the tolerance of atabrine by rats (Hegsted, McKibbin and Stare, '44a) led to the demonstration of a "choline-like" or "choline sparing" action of atabrine (Hegsted, McKibbin and Stare, '44b). Young rats receiving 40 mg. % of atabrine in the diet not only survived the acute stage of choline deficiency, but did not develop the hemorrhagic kidneys associated with this deficiency syndrome. This paper reports further experiments which demonstrate that atabrine has a "thiamine sparing" action.

EXPERIMENTAL[2] AND RESULTS

Thirty-six young male rats were fed a purified ration low in thiamine, half of the animals receiving the same diet plus 40 mg. of atabrine hydrochloride per 100 gm. of ration. The composition of the ration was the same as used in previous experiments (Hegsted, McKibbin and Stare, '44a) except that thiamine was lacking from the vitamin mixture. After 13 days the animals not receiving atabrine began to lose weight; they were then divided into six groups and fed thiamine supplements. The pertinent results are presented in table 1. It appeared that the rats receiving atabrine made much more efficient use of low levels of thiamine. However, the rations had been fed ad libitum and the animals not receiving the drug had gained somewhat faster and had lost a few grams in weight when supplements were started. Those receiving atabrine were still gaining. In order to rule out the effect of food consumption,

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[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

[2] We are indebted to Merck and Company, Rahway, New Jersey, for furnishing the crystalline-B-vitamins and the alpha-tocopherol, and to Abbott Laboratories, North Chicago, Illinois, for furnishing Haliver Oil.

TABLE 1

The effect of atabrine on the response to various levels of thiamine.

GROUP	ATABRINE IN RATION	WEIGHT AT END OF DEPLETION PERIOD	THIAMINE SUPPLEMENT	GAIN IN 30 DAYS	GAIN OF ATABRINE GROUPS COMPARED TO CONTROL
	mg. %	*gm.*	*µg. per day*	*gm.*	*%*
1	0	17.0	3	7.5	
2	40	18.5	3	23.8	318
3	0	18.5	6	36.0	
4	40	16.0	6	50.0	139
5	0	24.8	9	60.4	
6	40	19.2	9	54.4	90

the experiment was repeated and the food intake of the control animals limited to that consumed by the atabrine groups until their food intake fell below that of those receiving atabrine. After 21 days, the animals not receiving atabrine had lost considerable weight and were given thiamine supplements. Animals receiving atabrine, however, were continued on the basal ration without supplementation for 8 days longer. In spite of the longer depletion period, the atabrine groups gained more (1.78 gm. per day on 3 µg., and 2.36 gm. per day on 6 µg. per day) than the controls on the same levels of thiamine supplementation (1.20 gm. per day on 3 µg., and 2.26 on 6 µg. per day).

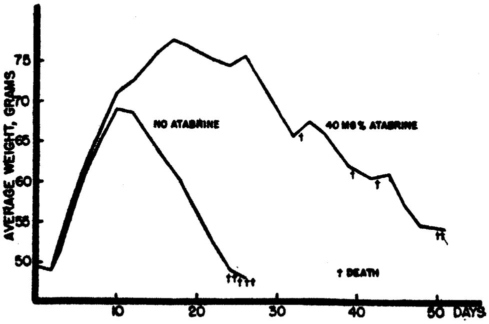

Fig. 1 The effect of atabrine on the growth of rats deficient in thiamine.

In a third experiment comparable groups of animals on the basal ration and ration plus atabrine were allowed to continue until death. As in the second experiment, the food intake was equalized until the tenth day when the intake of the controls fell below that of the atabrine group. The average growth curve for the animals and time of death are shown in figure 1. It is apparent that the course of the deficiency was much more acute in the animals not receiving atabrine. They lived an average of 25 days, whereas those animals receiving atabrine lived on the average of 43 days. Severe symptoms of polyneuritis were observed in most of the animals receiving atabrine while the controls usually died before showing clear-cut symptoms.

Thus it appears that regardless of the criteria used as evidence of deficiency (loss of weight, response to supplements, time of death), atabrine exerts a ''thiamine sparing'' action in young rats fed a ration low in thiamine. It will be noted in table 1 that this effect is more pronounced at the lowest level of thiamine intake, less so at the 6 µg. intake, and absent at the highest level of thiamine supplementation. This would be expected since it has been shown (Hegsted, McKibbin and Stare, '44a) that on a normal ration this level of atabrine inhibits growth.

SUMMARY

The addition of atabrine to a thiamine deficient diet for rats delays loss of weight, onset of symptoms, and death. Low levels of thiamine produce greater gains in animals receiving atabrine than in controls. Atabrine thus has a ''thiamine sparing'' action.

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ACUTE AND CHRONIC ASCORBIC ACID DEFICIENCIES IN THE RHESUS MONKEY [1]

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TWO FIGURES

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Signs of vitamin C deficiency in the Rhesus monkey (Macaca mulatta) have been described by many workers (Harden and Zilva, '18; Howe '23; Howitt, '31; Topping and Fraser, '39; Tomlinson, '39; Fraser and Topping, '42 and Tomlinson, '42). All these workers have emphasized the gingival changes which occurred.

Roff and Glazebrook ('39), Fitzsimmons ('41), Barahal and Priestman ('42), Kent ('43), Stuhl ('43) and Roth ('45) have described certain types of gingival changes in various groups of human beings which responded to ascorbic acid therapy. However, Crandon, Lund and Dill ('40) reported that in a case of acute scurvy experimentally produced in man, they observed no gingival changes. Pijoan and Lozner ('44), and Restarski and Pijoan ('44) believe that the assumption that gingivitis, with or without pyorrhea, is dependent upon a scorbutic basis is unwarranted unless there is antecedent or present clinical evidence of scurvy.

In our studies of ascorbic acid deficiency in the Rhesus monkey, very significant differences in the gross manifestations have been observed, depending on whether the deficiency was acute or chronic.

PROCEDURE

Ascorbic acid deficiency in the Rhesus monkey was produced on a ration composed of: sucrose 74%, acid-washed casein 18%, salts IV (Phillips and Hart, '35) 4%, and cottonseed oil 4%; 1:20 liver extract was added at a level of 3% at the expense of the entire ration. The daily

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We are indebted to Merck and Co., Rahway, New Jersey, for the synthetic vitamins; to Abbott Laboratories, North Chicago, Illinois, for halibut liver oil; and to Wilson Laboratories, Inc., Chicago, Illinois, for the 1: 20 liver extract.

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vitamin supplement consisted of: thiamine 1 mg., riboflavin 1 mg., pyridoxine 1 mg., calcium pantothenate 3 mg., nicotinic acid 5 mg., choline 50 mg., inositol 100 mg., and para-aminobenzoic acid 100 mg. The supplement was given in 40–50 ml. of dilute sucrose solution which was placed in the water cup prior to feeding and watering. Once a week 0.5 ml. of halibut liver oil fortified to contain 500 I.U. of irradiated ergosterol was given orally to each monkey. Two control monkeys no. 129 and no. 130, were given the above ration and vitamin supplements plus 7.5 mg. of ascorbic acid per kilogram of body weight daily.

Four young monkeys (2 males, no. 100 and no. 102, and 2 females, no. 97 and no. 98) were used. Their weights ranged from 2.5 to 2.7 kg. and they were between 1½ and 2 years old. When an acute ascorbic acid deficiency appeared, a single dose of crystalline ascorbic acid, varying from 15 to 100 mg., was given to measure the ability of the acutely deficient monkey to respond to therapy. When the acute deficiency signs began to reappear, crystalline ascorbic acid was given daily at a level of 0.25 mg. per kilogram of body weight as a means of producing a chronic ascorbic acid deficiency. This subminimal level of ascorbic acid was continued for a period ranging from 80 to 100 days. When no. 100 was sacrificed for histological examination, the other three were given 7.5 mg. of ascorbic acid per kilogram of body weight, per day.

Later, two young male monkeys (no. 168 and no. 169) were used to study the complicating effects of biotin in the chronic stage of ascorbic acid deficiency. No. 168 received the same diet as those in the previous experiment, while no. 169 received in addition a daily supplement of 20 μg. of Merck's crystalline biotin. A second period of chronic ascorbic acid deficiency was produced in no. 98 during which 20 μg. of biotin were given orally.

Periodic examinations were made of the structures of the oral cavity. When a period of ascorbic acid deficiency began, bi-weekly examinations were necessary. At the end of these studies, the monkeys were sacrificed for histological examination.

RESULTS

The four monkeys which did not receive any ascorbic acid developed an acute deficiency in 30 to 50 days. The growth curves for these animals are shown in figure 1. The first sign of an imminent deficiency was the gradual loss of about 10% of the body weight over a period of approximately 2 weeks. In the next week, a more precipitous loss of an additional 10 to 15% of the original body weight occurred.

The coat of the acutely deficient monkeys became very rough without any loss of hair. Appetite decreased very rapidly during the period of weight loss. Even when the acute deficiency was allowed to become very severe, no gingival changes were observed in any of the four monkeys but the tooth surfaces were covered with a heavy residue.

One of the most profound changes observed, which paralleled the loss of weight in severity, was the apparent unwillingness of the monkeys to use their hind legs. This was first seen about a week after the precipitous weight loss began. The monkeys rarely left the bottom of the cage and locomotion consisted of the monkey pulling itself around by means of its arms. An acute tenderness of the joints, especially of the knees, was observed. When test doses of ascorbic acid were given, the appetite of the monkeys increased immediately and within a week the locomotor ability and the appearance of the hair coat was normal.

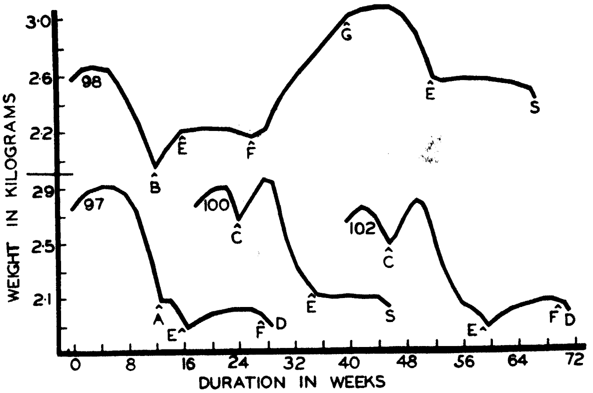

Fig. 1 Growth curves of four monkeys during period of acute and chronic ascorbic acid deficiencies.

A. 15 mg. of ascorbic acid given.
B. 30 mg. of ascorbic acid given.
C. 100 mg. of ascorbic acid given.
E. Began 0.25 mg. of ascorbic acid per kilogram of body weight per day.
F. Increased ascorbic acid to 7.5 mg. per kilogram of body weight per day.
G. Discontinued ascorbic acid therapy.
D. Died.
S. Sacrificed.

When the acute deficiency signs began to appear again, they were identical with those in the first period. Since the appetite of the animals decreased as the acute deficiency signs appeared and did not improve when 0.25 mg. of ascorbic acid per kilogram of body weight was given per day, 3 gm. of 1:20 liver extract were given to each monkey daily by mouth. In this way the animals were supplied with these essential nutritional factors, normally supplied by the 1:20 liver extract contained in the ration.

The tenderness of the hind legs observed in the acute deficiency became rapidly worse in the chronic state. This was seen especially in no. 100 and no. 102. After 4 and 6 weeks, respectively, the soreness about the knees was extreme. Later in no. 100, a complete separation of the proximal end of the right tibia from the epiphysis was observed. The bony shaft of the tibia was located just under the skin in the region of the patella. In no. 97 and no. 98, the soreness became very great, but no separation was noted grossly.

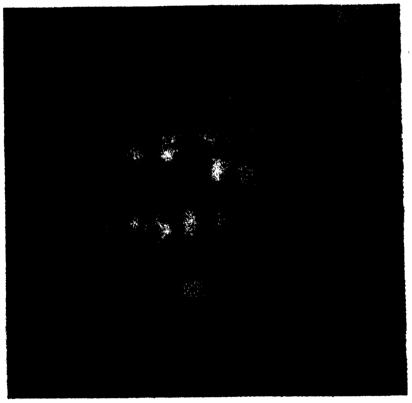

Fig. 2 Late gingival changes in a chronic ascorbic acid deficiency. Hypertrophy and necrosis of gingiva and shifting of the deciduous molars.

For the first 10 to 14 days of the chronic deficiency, no changes were observed in the gingiva. Subsequently in all four monkeys, there was observed a swelling and hyperemia of the gums, especially in the interdental papillae. This condition became progressively worse with the hyperemia spreading to the buccal mucosa (fig. 2). The gums bled very easily and spontaneously as the chronic deficiency progressed. Pressure on the teeth, either in mastication or when applied during an oral examination, indicated a definite tenderness. When the spontaneous bleeding was most pronounced, there was necrosis of the gums, particularly of the interdental papillae. This necrosis spread until it involved large areas of the gums. Some alveolar resorption occurred which resulted in a loosening of the teeth and a premature loss of some of the deciduous molars.

When the gingival lesions began to appear, the white, sticky residue on the teeth observed in the acute deficiency became thicker, especially on the surfaces bordering the gums. As the gingival lesions progressed, these heavy deposits were replaced in some areas by thick regions of tartar.

Late in the chronic deficiency period, a severe loss of hair was observed over the entire body, particularly on the belly and hind legs. The skin was usually very dry with small regions of dermatitis on the face and belly. A mild edema occurred occasionally about the groin, upper legs and around the eyes. Hemorrhage occurred frequently about the eyes and more rarely on the body and extremities.

After 80 days of chronic deficiency, monkey no. 100 was sacrificed for histological examination. After 80 to 100 days of chronic deficiency, nos. 97, 98 and 102 were given 7.5 mg. of ascorbic acid per kilogram of body weight daily. Nos. 97 and 102 did not respond to ascorbic acid therapy but died within 2 weeks. No. 98 responded gradually; after a month of therapy, there had been a moderate increase in weight, a great increase in activity and a loss of soreness about the joints. However, there had been no improvement in the hair coat. At that time, 20 µg. of crystalline biotin were given daily. After 6 weeks, a new growth of hair began which developed until the monkey was completely covered.

The gingival lesions in no. 98 began to recede in the third week of therapy. The necrosis and bleeding were greatly reduced and finally disappeared. The gums never completely returned to their normal size and color but remained hypertrophic and hyperemic, especially in the regions where the most extensive necrosis had occurred and where deposits of dental tartar still remained. Sufficient necrosis had occurred in the lower right quadrant to cause a shifting of the deciduous and first permanent molars which persisted.

In the study of the effect of biotin upon the chronic ascorbic acid deficiency, the monkeys (nos. 98 and 169) which received 20 µg. of biotin per day maintained a normal amount of hair which became rough and unkempt. However, no. 168 exhibited a similar hair loss to the previous group of chronically deficient monkeys. The addition of biotin made no other difference in the signs of the chronic ascorbic acid deficiency.

Throughout these experiments, the control monkeys maintained a normal rate of growth comparable to those fed 3% 1:20 liver extract by Waisman et al. ('43). No gingival lesions, skin changes or hair loss were observed.

When the monkeys were sacrificed, post-mortem examinations were made. The skeletal changes were the most pronounced. In the six chronically deficient monkeys, a very great increase in the size of the costochondral junctions was observed. In nos. 97, 100 and 102, there had been a complete separation of the proximal end of the tibia from the epiphysis. The proximal end of the tibia was very pointed and porous. The distal end of the femur was less affected, and no complete separation from the epiphysis was seen. Large areas of hemorrhage were observed between the epiphysis and the ends of the tibia and femur. Nos. 98, 168 and 169 showed similar but less extensive changes in the tibia and femur.

There had been considerable alveolar bone resorption in the areas of most extensive necrosis of the gingiva. This was most evident on the crest of the lateral plates. Extensive bone resorption was also observed over the exterior surface of the cranium and on both surfaces of the rami of the mandibles.

In monkeys nos. 97, 168 and 169 numerous carious lesions were observed in the first permanent molars. No lesions were observed in any of the deciduous teeth. In no. 97 there were 8 lesions: 3 in each upper molar and one in each lower molar. Thirteen lesions were observed in no. 168: 4 in each upper molar, and 2 and 3, respectively, in the left and right lower molars. Eight lesions were observed in no. 169: 4 and 2, respectively, in the left and right upper molars, and one in each lower molar. No carious lesions were observed in the other chronically ascorbic acid deficient monkeys, nor in the two control monkeys.

DISCUSSION

Two distinct syndromes of ascorbic acid deficiency have been observed in the Rhesus monkey depending on whether the deficiency was acute or chronic. An acute deficiency was characterized by a precipitous weight loss and tenderness in the joints of the legs but no gingival lesions. A chronic deficiency was characterized by severe gingival lesions and skeletal changes but no rapid decrease in weight.

The signs of a chronic ascorbic acid deficiency are very similar to those described by the workers who produced an ascorbic acid deficiency in monkeys by the use of natural rations (Harden and Zilva, '18; Howe, '23; Topping and Fraser, '39; Fraser and Topping, '42 and Tomlinson, '42). The gingival lesions are especially similar to those described in scorbutic monkeys by Tomlinson ('39), by Fraser and Topping ('42) and by Tomlinson ('42) and in scurvy in man by Hess ('20).

Roff and Glazebrook ('39), Fitzsimmons ('41, '42), Barahal and Priestman ('42), Kent ('43), Stuhl ('43) and Roth ('45) have described gingival lesions in man which receded when ascorbic acid was given. These authors stated that there were other cases of periodontal disease which did not respond to ascorbic acid therapy. The gingival lesions which did respond to therapy appeared to be similar to the gingival lesions described in classical scurvy in man (Hess, '20) and in chronic ascorbic acid deficiency in the Rhesus monkey.

Recently Crandon, Lund and Dill ('40) and Restarski and Pijoan ('44) have described cases of experimental scurvy in man, in which they observed no gross changes in the gums or teeth. Pijoan and Lozner ('44) and Restarski and Pijoan ('44) believe that the assumption that gingivitis rests on a scorbutic basis is unwarranted unless there are accompanying clinical evidences of late scurvy. These experimental cases of scurvy in man are similar to the acute deficiency in the monkey where no gingival lesions were observed.

Since there are such great differences in the manifestations of the acute and chronic phases of an ascorbic acid deficiency in the monkey, it is necessary to consider these differences when scurvy in man, experimental or spontaneous, is being studied and described.

Howe ('23, '27) reported a high incidence of carious lesions in scorbutic monkeys which were fed a natural diet. We have observed caries in three of six chronically ascorbic acid deficient monkeys but none in the two controls. The number of animals is too small to consider the caries incidence significant. However, the frequency of carious lesions in young monkeys which have been on experiment for only 8 months is usually so low that a 50% incidence in this small group and the high number of lesions per animal may indicate that the caries index is increased in a chronic ascorbic acid deficiency.

SUMMARY AND CONCLUSIONS

An acute ascorbic acid deficiency was produced in young Rhesus monkeys in 30 to 50 days. In the acute phase there was a precipitous loss in weight, slight tenderness of the joints and heavy residues on the tooth surfaces. However, no gingival changes were observed. When a single test dose of ascorbic acid was given, there was a very rapid and complete alleviation of these signs.

In the chronic state of ascorbic acid deficiency, a series of gingival changes was observed which was accompanied by increasing food deposits on the teeth and dental tartar. There was very marked tenderness and soreness at all the joints accompanied by a considerable swelling. Bone resorption occurred at the bone-cartilage junctions, especially at the ends of the long bones of the legs. There was a very extensive loss of hair and a mild dermatitis; both could be prevented or cured by crystalline biotin. In three of the six chronically deficient monkeys, there was a high incidence of dental caries.

These data indicate that the signs of acute and chronic ascorbic acid deficiencies in the monkey are distinctly different. They may partially explain the differences observed in experimentally produced and spontaneous scurvy in man.

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THE AVAILABILITY OF VITAMINS FROM YEASTS

I. THE ABSORPTION OF THIAMINE BY HUMAN SUBJECTS FROM VARIOUS TYPES OF BAKERS' YEAST[1]

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It was shown as early as 1933 by Walker and Nelson that rats were not able to utilize thiamine from fresh yeast so well as that from dried yeast containing fewer viable cells. This was confirmed by Parsons and Collord ('42). Parsons, Collord, Strong and Peterson ('42) and Parsons and Collord ('42) found that boiled yeast was a better source of thiamine for human subjects than fresh unboiled yeast. It was shown that thiamine not accounted for by the urinary excretion during the periods of fresh yeast ingestion was largely present in the feces so that the failure of the urinary thiamine to rise was attributable to lack of absorption.

Opportunity was afforded for studying simultaneously with human subjects and with rats the absorption of thiamine from several types of bakers' yeast; these proved to represent three levels of thiamine concentration, as well as some diversity in regard to the content of other vitamins. The results of the human studies are presented in this paper;[4] supporting data on the same yeasts when fed to rats are given elsewhere (Parsons et al., '45).

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[1] Published with the approval of the director of the Wisconsin Agricultural Experiment Station. Certain of the results have appeared in abstract form in the Proc. of the Fed. of Am. Soc. for Exp. Biol. for 1944, vol. 3, p. 96. This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

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[4] Generously supplied by Eli Lilly Co.

EXPERIMENTAL

Determinations are reported of the urinary thiamine eliminations of twenty-three human subjects (fecal, of eleven) on controlled basal diets to which were added, in test periods of 3 to 5 days, samples of compressed bakers' yeasts typical of those found on the market (see table 1 of following paper by Parsons, Foeste and Gilberg, '45). Yeasts A and C were of different strains and contained 0.5 mg. of thiamine per cake. In a recently introduced modification of yeast A, designated here as A_2, the thiamine content was doubled, although this change was in no way evident from the label. Yeasts C, D, D_2 and E were made from the same batches as were corresponding samples of yeast F but were modified by the manufacturers not only by increases in their thiamine content (30-fold for yeast C and 250- to 300-fold for D, D_2 and E) but also in certain other respects. Their manufacture will be further discussed below. Yeasts A and A_2 were procured for the experiment on the open market. The other samples were furnished by the manufacturers in weekly shipments. The extent to which the various yeasts would release thiamine available for absorption during digestion was tested by feeding them both fresh (as purchased) and after boiling. The method of preparing the heated yeast was as follows: The fresh yeast was broken up in a small amount of water and the suspension was poured into rapidly boiling water which was maintained at the boiling point for 10 to 120 seconds and consumed after cooling. The uncooked yeast dose was suspended in an equal quantity of cold water. In the case of yeast A, the uncooked yeast dose was also consumed, in some instances, suspended in tomato juice (diet 1) or pineapple juice (diet 2) or eaten as the solid cake. Such variations in these and in some additional experiments (Link) had no effect on the usual low output of urinary thiamine or the appearance of live yeast cells in the feces. In control periods, thiamine hydrochloride [5] was substituted for the yeast sources in equivalent amounts.

Two basal diets were employed: basal diet 1, an ordinary type of diet similar to that described by Parsons and Collord ('42), and basal diet 2, a dairy products-pineapple-bread diet (Parsons, Stettler, Williamson and Johnson, '44). Further details of the experiment are presented in the tables. In all cases, the basal diet supplied sufficient thiamine to meet the metabolic requirements of the subjects and, hence, urinary excretions were considered to reflect the absorption of thiamine furnished by supplements. However, as a special precaution some subjects were given thiamine hydrochloride previous to the experiment (table 3). The subjects comprised graduate or senior women of the

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[5] Generously supplied by Eli Lilly Co.

University of Wisconsin and were judged to be in a satisfactory nutritional state.

Subjects of study 1 participated in two test diet periods separated by an interval of 60 days, during which yeast D was ingested in the fresh form. The plan of this experiment originated in the suggestion of other workers that the prolonged ingestion of fresh yeast would enable the body to become so habituated to it that the availability of the thiamine would be improved. Yeast D was chosen for this test inadvertently on the assumption that thiamine was as poorly absorbed from this type as it had been from the other yeasts investigated. Only after the 60-day interval of fresh yeast ingestion was in progress was it realized from the results of urine assays from the previous period that yeast D, in the fresh form, supplied available thiamine; hence, instead of a test for habituation to raw yeast, the interval served to load body stores with thiamine to the extent that urinary excretions for all subjects were significantly higher during the second test period (table 1).

TABLE 1

Thiamine intake and average daily urinary and fecal thiamine elimination of subjects consuming yeast D both fresh and after treatment by boiling.

STUDY 1[1] (3 SUBJECTS)						STUDY 3[1] (1 SUBJECT)				
Period	Thiamine intake		Thiamine output			Period	Thiamine intake		Thiamine output	
	Basal[2]	Supplement	Urinary excretion		Fecal output		Basal[3]	Supplement	Urinary excretion	Fecal output
			test I	test II	test I					
	mg./day	*mg./day*	*µg./day*	*µg./day*	*µg./day*		*mg./day*	*mg./day*	*µg./day*	*µg./day*
Basal (4 days)	2.0	..	440	520	500	Basal (5 days)	1.0	..	315	115
Boiled yeast (4 days)	2.0	3.7	930	1335	800	Fresh yeast (5 days)	1.0	2.0	560	605
						Basal (3 days)	1.0	..	285	160
Fresh yeast (4 days)	2.0	3.7	1000	1420	880	Boiled yeast (5 days)	1.0	2.0	625	645
Thiamine hydrochloride (4 days)	2.0	3.7	....	1750	...	Thiamine hydrochloride (5 days)	1.0	2.0	610	420

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[1] Biological assay of thiamine, study 1; chemical assay, study 3.

[2] Basal diet 1 to which was added 1.0 mg. crystalline thiamine hydrochloride per day throughout the four periods.

[3] Basal diet 2 including 600 gm. canned pineapple juice per day.

One subject of study 3 (table 2) consumed, for 3 days, yeast A treated by freezing; when it became apparent from the assays that the absorption of thiamine from this form of yeast was not improved over that from the fresh form, the yeast was fed after boiling as a control test for 1 day; even to this brief period the subject responded promptly with increased urinary thiamine excretion.

TABLE 2

Thiamine intake and average daily urinary and fecal thiamine eliminations of subjects of groups 2 and 3 consuming yeasts A, C or E both fresh and after treatment by boiling.

	STUDY 3								STUDY 2			
Period	Thiamine intake		Average daily thiamine output						Length period	Thiamine intake		In urine yeast A (3 subj.)
			Yeast C (3 subj.)		Yeast E (3 subj.)		Yeast A (3 subj.)					
	Basal[1]	Supplement	Urine	Feces	Urine	Feces	Urine	Feces		Basal[2]	Supplement	
	mg./day	*mg./day*	*µg.*	*µg.*	*µg.*	*µg.*	*µg.*	*µg.*	*days*	*mg./day*	*mg./day*	*µg./day*
Basal (5 days)	1.0	..	200	225	210	260	155	450	3	1	..	130
Fresh yeast (5 days)	1.0	2.0	185	1115	295	350	100[3]	2625	3	1	1	80
Basal (3 days)	1.0	..	190	740	200	150	150	220	..	..	..	..
Boiled yeast (5 days)	1.0	2.0	670	315	670	160	525	...	3	1	1	330
Thiamine hydrochloride (5 days)	1.0	2.0	880	485	880	130	885	610	3	1	1	290

[1] Basal diet 2 including 600 gm. canned pineapple juice.

[2] Basal diet 1.

[3] One subject of this group in a 3-day period consumed the fresh yeast after it had been frozen for 24 hours. See discussion of results.

All thiamine determinations were made by the thiochrome method, essentially that of Hennessey ('42). Duplicate determinations were made on separate days for individual samples. Periodic recoveries of standard amounts of thiamine added to the samples averaged 95% and were for the most part within the range 92–96%. Food aliquots representing 10% of the weighed daily food consumption were homogenized and assayed in the fresh form for thiamine.

RESULTS AND DISCUSSION

Two yeast samples with comparable thiamine content, A and C, gave results in agreement with earlier data on yeast A and B (Parsons and Collord, '42). Apparently, these yeasts contributed little or no thiamine to the body when fed to human subjects in the amounts ordinarily recommended (yeasts A and C, table 2; yeast C, table 3). It even appears that thiamine from the basal diet was taken up to an appreciable extent by these types of fresh yeast and so bound that it became largely unabsorbable in the digestive tract. This was indicated by the consistently diminished urinary thiamine excretion during the fresh yeast period as contrasted to the preceding basal period whether the amount of yeast ingested was two or four cakes. The average decrease in the urinary thiamine output during the period of fresh yeast feeding in comparison with the preceding basal period was, for the six subjects on yeast A, 37%; for the six subjects on yeast C, 17%. This effect of fresh yeast seemed to carry over slightly into the succeeding short basal period for those subjects for whom this period was provided. Although alternative explanations for the lessened urinary thiamine in the fresh yeast period might well be offered, the fecal thiamine values tend to substantiate the hypothesis stated above.

Thiamine from yeast D, in contrast to that from yeasts A and C, was nearly as available from the fresh as from the boiled yeast during duplicate tests in study 1 and also in a later test with another subject (study 3, table 1). That this difference was valid and fundamental was confirmed by the results of animal assays (described in the following paper) which gave uniformly high values for the utilization of fresh yeast D for all assays during the 15-month interval between the two human experiments above. Unexpectedly, however, the human and rat tests for the extent of absorption of thiamine from this fresh yeast simultaneously showed a lower assay value than formerly in comparison with boiled yeast D or with equivalent thiamine hydrochloride. So unmistakable was the change that this new form of the yeast was designated yeast E (see table 2) and information on possible alterations in its production was sought from the manufacturers. It was discovered that, beginning with the date on which the change in assay value occurred, yeast D had been taken off of the market; the sample which was sent to this laboratory was made in small amounts as a special courtesy and only thiamine hydrochloride added instead of other substances as well (see table 1 of following paper). However, in a new sample, the manufacturers scrupulously reproduced every step of the former procedure for yeast D, as far as possible. This sample (yeast

D_2) fed fresh yielded very nearly the same assay values as had yeast D (table 3). The procedure in the tests with yeast D_2 was such that the results removed the possibility that the difference in the urinary outputs on yeasts D and E might have been due to the change in sequence of periods or the total intake of thiamine or of yeast. The strain of yeast was not an essential factor in explaining the difference between E and D_2, inasmuch as yeast C, from which D_2 was made, lowered the urinary thiamine when fed fresh as it had in the earlier tests (tables 2 and 3). Laxation produced by the various yeasts was ruled out as a decisive factor causing the differences in absorption of the thiamine from them by results recently published elsewhere (Parsons, Stettler, Williamson and Johnson, '44; Stettler, '44).

TABLE 3

Thiamine intake and average daily urinary output for subjects consuming yeast A_2, C or D_2.

Period	STUDY 4				STUDY 5					
	Thiamine intake		Thiamine in urine		Thiamine intake		Thiamine in urine			
	Basal[1, 2]	Supplement	Yeast D_2	Yeast A_2	Basal[1]	Supplement	Yeast D_2		Yeast C	
			2 subj.	2 subj.			1 subj.[3]	1 subj.	1 subj.[3]	2 subj.
	mg./day	*mg./day*	*avg. µg.*	*avg. µg.*	*mg./day*	*mg./day*	*avg. µg.*	*avg. µg.*	*avg. µg.*	*avg. µg.*
Basal (4 days)	2	.	565	590	1	.	215	290	310	295
Fresh yeast (4 days)	2	2	1080	850	1	2	465	610	180	245
Basal (3 days)	.	.	...	...	1	.	275	435	215	...
Boiled yeast (4 days)	2	2	1380	1505	1	2	550	...	550	...
Thiamine hydrochloride (4 days)	.	.	...	...	1	2	655	...	655	...

[1] Basal diet 2 including 600 gm. whole crushed canned pineapple per day. Small, fixed increases in the thiamine intake of individual subjects resulted from slight dietary modifications necessitated by controlling protein intake.

[2] 1.0 mg. of thiamine hydrochloride daily was added to the basal diet of group 4 throughout the three periods.

[3] Given 3.0 mg. of thiamine hydrochloride for 4 days before the experiment; 2 days elapsed without the dose before the first basal period.

In the meantime, during a routine re-assay of the thiamine of yeast A it was discovered that the manufacturers had modified this yeast by doubling the thiamine content as well as by the changes declared on the label (table 1 of the following paper). Hence, this new yeast, designated as yeast A_2, was fed to two members of a diet squad (study 4, table 3). It did not behave as yeast A had in former repeated trials with respect to decreasing the output of urinary thiamine in comparison with a preceding basal period, although, as in the case of E, the absorbability of its thiamine was still much improved by boiling the yeast. The relative urinary increase of thiamine due to the intake of fresh yeast A_2 was somewhere between that of yeast E which had a higher thiamine content and yeast D which had, in addition, a higher content of other factors. This would be in harmony with an hypothesis that various kinds and concentrations of substances added to the yeast cell may influence the readiness with which the cell gives up its thiamine in the digestive tract. Some specially prepared commercial yeast samples are being studied at present in an investigation of this theory.

It is evident that subjects receiving D, D_2 and E as the thiamine supplements were required to take much smaller amounts of yeast to supply the desired thiamine intake than were subjects ingesting the types with a lower thiamine content. It might reasonably be postulated, therefore, that this smaller amount of yeast would be more vulnerable to the digestive processes and, hence, offer less difficulty in releasing its thiamine high up in the digestive tract. That the influence of the amount of yeast was negligible, however, within the limits of the experiment and that other factors played a more important role is evident from the data. For example, the feeding of yeast A, fresh, in the amounts of either 2 or 4 cakes (about 25 or 50 gm.) unfailingly resulted in reducing the urinary output of thiamine in comparison to that on the same diet without it. Fifty grams of fresh yeast A_2, however, increased measurably the urinary output of thiamine instead of decreasing it. As little as 8 gm. per day of yeast E was only fairly well utilized in contrast to 8–18 gm. of yeast D and D_2 from which the thiamine was apparently more readily absorbed.

The generalization in a preliminary report from Melnick ('44) that only about 16% of the thiamine in fresh yeast is available to human subjects cannot be discussed in relation to the present results until fuller publication is available and the identity of the type of yeast in Melnick's experiment is known.

Preliminary experiments in this laboratory on the fecal elimination of live yeast cells have been confirmed and extended. Consistently negative results were obtained when the subjects consumed boiled yeast or no yeast, but positive results whenever fresh yeast was consumed. Assays of total 24-hour collections of fecal material for periods of several days following the ingestion of 50 gm. of fresh yeast showed a prompt elimination of a considerable number of living yeast cells and the persistence of a diminishing number of 1 or more days following this. Carmine was ingested to mark the beginning of the fecal period, and the dose of yeast was consumed several hours later. Following are the total daily values obtained for two subjects expressed as the number of thousand viable yeast cells eliminated on consecutive days: For subject M.L.J., yeast A, 565000, 182 and none; yeast D_2 none, 5122, 19, none and none; for subject C.M., yeast A, 177 and 46; yeast D_2, 15, 148000, 110, 31 and 64. As the highest elimination represented only between 1 and 2% of the total number of yeast cells consumed, it is, perhaps, not surprising that no quantitative relationship was seen between viable yeast cells and the relative urinary excretion of thiamine on the two yeasts, the latter possibly being dependent on rapid absorbability of thiamine at a fairly restricted segment of the digestive tract while destruction of yeast cells presumably persisted far beyond such an area.

In agreement with former results, it has again been found true that the amounts of thiamine in urine and feces on certain fresh and boiled yeasts show a somewhat reciprocal relationship (yeasts A and C, table 2). It appears that the failure of the urinary thiamine level to rise in response to the ingestion of thiamine in the form of fresh yeasts A and C was chiefly due to lack of absorption of the vitamin from the intestinal tract, inasmuch as the fecal thiamine content was significantly lower when thiamine hydrochloride or these yeasts in the boiled form were fed, than when the yeasts were fed fresh. It may be noted that one subject (table 2, study 3, yeast A) eliminated 2.6 mg. of thiamine per day in the feces during the fresh yeast period. Although this amount was usually high, somewhat comparable amounts (1.9 mg. avg. per day) had been reported previously with the same type of yeast (Parsons and Collord, '42).

CONCLUSIONS

1. Two types of bakers' compressed yeast, fed fresh to human subjects were poorly utilized as sources of thiamine as indicated by low urinary and high fecal eliminations of thiamine. The ingestion of these yeasts appears even to have decreased the amount of available thiamine supplied by the basal diets, inasmuch as the urinary thiamine was reduced below that of the preceding basal period and fecal thiamine

was much increased. Living yeast cells were recovered from the feces after fresh yeast feeding.

2. Marked improvement in thiamine absorption from these yeasts was obtained by boiling them for brief periods.

3. Four fresh yeast samples, manufactured by the same two firms that prepared the first two types described but with higher vitamin content, did not show the previously observed effect of lowering the urinary output of thiamine in human subjects but led to varying degrees of increased thiamine excretion instead; the yeast most notable in this regard was the one with the highest vitamin levels. The explanation for this apparent correlation has not been determined.

4. The differences in the absorbability of the thiamine from various fresh yeasts did not seem to be attributable either to the strain of yeast or to the amount of yeast consumed by the subjects.

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THE AVAILABILITY OF VITAMINS FROM YEASTS

II. THE ACCESSIBILITY TO RATS FOR GROWTH OF THE THIAMINE IN VARIOUS TYPES OF BAKERS' YEAST [1]

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ONE FIGURE

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The availability to human subjects of thiamine from yeasts just reported (Parsons, Williamson and Johnson, '45) was judged on the somewhat indirect although acceptable evidence of the presence of additional thiamine in the urine following doses of yeast and a corresponding absence of a striking increase in the feces. The indication thus obtained of differences in the absorbability of the thiamine of various yeasts has been strengthened by similar observations of differences in utilization obtained by the more direct evidence of variations in the body weight of rats on given doses in biological assays carried out simultaneously, to be reported in this paper.

EXPERIMENTAL

The various compressed bakers' yeasts studied in these experiments are identified in table 1. All doses of yeast were administered separately instead of being incorporated in the basal ration. This was particularly desirable in view of the pronounced change in odor and taste of the yeasts under various treatments which, in itself, might otherwise have influenced the intake of the ration. A high palatability of the basal diet was also sought. The percentage composition of the ration selected, adapted from one used by Schlutz and Knott ('36, '39) was: purified casein 15; fresh pig liver autoclaved at 120° for 5 hours and dried 15; sucrose 48.5; Crisco 17; and salt mixture (Osborne and Mendel, '19) 4.5. All rats received a vitamin supplement containing the following per rat per week: riboflavin 200 μg.; choline 35 mg.; nicotinic acid 170 μg.;

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[2] Present address: Dietitian with the armed forces in France.

calcium pantothenate 1.5 mg.; pyridoxine 200 μg.; and 6 drops of cod liver oil. This supplement was distributed in several doses per week or, in the instance of pyridoxine, was incorporated in the basal ration for some rats.

The preparation of yeast doses for feeding consisted in suspending a weighed portion in water and making it up to a suitable volume. The individual doses of yeast and vitamin supplements were fed directly to the rat by pipette except when the eagerness of the rat for the dose made this difficult to manage, in which case the measured dose was offered in a small feeding dish and the rat was watched for the few moments necessary for the quantitative ingestion of the dose.

In preparing the liver, an autoclave was used which allowed the reading of temperatures directly rather than by pressure gauge, inasmuch as it was believed by Knott [3] that precision in heat treatment is necessary for a reliable degree of destruction of thiamine without too great injury to other nutritive factors. Assurance of the satisfactory absence of thiamine from the basal ration was given by the regularity and swiftness of the depletion of the negative control rats, i.e., a sharp cessation of growth at the end of 7 to 12 days during which there was an average total gain of 34.6 gm.; and death after typical convulsions on an average of 32 days (range, 28 to 36 days) and an average total weight loss of 41.8 gm. A dose of 4 μg. of thiamine hydrochloride [4] per day produced an average gain of 1.5 gm. body weight for the positive control rats of 150 gm. or less. Beyond this weight, the dosage was raised to 5 and 6 μg. for rats up to 250 gm. body weight, for about the same rate of gain. The level of intake of basal ration of these rats on such levels of thiamine supported apparent vigor and the absence of abnormalities during the 16 to 32 weeks of the experiment except for some brown scurf under the thick hair along the back and a barely detectable amount of reddish stain on the hair around the nose and on the forepaws.

In conformity with the Schlutz and Knott's ('36) method, a short feeding period (10 to 15 days) was adopted for several reasons. For example, although the composition of the basal ration was presumably unfavorable to extensive intestinal synthesis, the short period was thought to be an added precaution against any tendency for fresh yeast, fed constantly over long periods, to become established in the rats' digestive tract or to encourage some special type of bacteria, which might conceivably obscure the assay through synthesis of thiamine. Human subjects in this laboratory showed living yeast cells in the feces for several days after a single dose of fresh yeast (Parsons, Williamson and Johnson, '45). In actual tests, however, a somewhat contrary tendency was frequently observed in the rats held for several weeks on fresh yeast doses at high levels which seemed at first to be quite adequate, namely, an unaccountably sharp and extensive decline after vigorous growth. This type of curve was not observed on doses of thiamine hydrochloride or boiled yeast and, hence, was difficult to evaluate in the assay, making long periods and high dosage of doubtful assay value. In addition, the short period was well adapted to the detection of possible changes in the potency of the manufactured yeasts, since speedy identification of any such changes with even slight alterations in procedure in the factory might yield valuable data.

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[3] Personal communication.

[4] Generously supplied by Eli Lilly Company.

A uniform dosage was used in both of the alternating periods of fresh and treated yeast samples. This was done not only because of the difficulty, just mentioned, with the higher doses but also for the purpose of avoiding diverse levels of intake of other factors from so potent a material as yeast. Even though a level of dosage satisfactory in the case of treated yeast often led to losses of body weight when fresh yeast was fed, nevertheless, the satisfactory gains in weight in the following period on treated yeast appeared to justify the procedure.

The three treatments of the yeast used were selected from the standpoint of their effect on the viability of the yeast cells. One treatment consisted in suspending yeast in about 5 parts of 80% ethyl alcohol for 24 hours, then reducing it to dryness before a fan at room temperature. Other samples were suspended in 3 parts of water and brought rapidly to boiling which was maintained for 10 to 120 seconds.

Prolonged periods of freezing such as are known to kill yeast cells had been found in another laboratory[5] to be effective in increasing the utilization of the thiamine of yeast for rats. Inasmuch as lengthy periods would be impractical as a household means of rendering the thiamine of yeast more available for absorption, shorter periods were tested. Cakes of yeast in their individual wrappers were put into the freezing unit of a household electric refrigerator at — 5°C. and left for 24 hours. In an attempt to increase the disruption of the yeast cell during freezing and thawing, other samples were suspended in 5 times their weight of liquid. A nutrient solution was used, inasmuch as this was being employed in other experiments on yeast at the time. It contained 1.0 gm. di-ammonium sulfate and 50.0 gm. anhydrous dextrose per liter. The doses of frozen yeast were fed either immediately after thawing or 1 hour later. In plate counts carried out subsequently, it was established that none of these procedures was significantly injurious to the yeast cell.

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[5] Personal communication from Prof. N. B. Guerrant, Department of Biological Chemistry, Pennsylvania State College.

RESULTS

The wide range of variation shown in the availability of thiamine of different yeasts consumed fresh by human subjects (Parsons, Williamson and Johnson, '45) was noted also when these same yeasts were fed to rats. As in the human experiments, here also there were only two yeast samples, i.e., those with the highest vitamin content, yeasts D and D_2, which appeared to be about equally effective sources of thiamine whether fed fresh or after boiling or treatment with alcohol; the availability of the thiamine of the other six samples fed was much lower and was significantly increased by these means. One of these six samples, F, was not fed to human subjects in our experiments because of the limiting low thiamine content (table 1) but from its position in the series with respect to C, D and E, its low thiamine availability and the improvement in this due to boiling would be expected.

With the particular technic used in these experiments for comparing the accessibility of thiamine from fresh as contrasted with treated yeasts in the digestive tracts of rats, minor variations between yeasts such as that between yeasts A and A_2 were not clearly demonstrated as they were in the human experiments. This seems reasonable when it is considered that the basal diet of the rat was essentially devoid of thiamine, whereas, much of the minor variation apparent in the effect of the various fresh uncooked yeasts on the urinary output of thiamine of human subjects appeared to depend on the extent of influence of the fresh yeast on the thiamine of the ingested food. Experiments aimed at reproducing the dietary conditions of the human experiments more closely in the rat are under way in this laboratory.

Boiling the poorly utilized fresh yeast samples and soaking them in alcohol were equally effective in improving the availability of the thiamine as seen in comparable tests in different groups of rats and in alternate periods of the same rats (fig. 1, groups C 2 and C 3). This similarity of effect of the two processes might be expected from the equal destruction of viability of the cell which they entailed. The short periods of freezing, on the other hand, were practically non-injurious to the cell, and, correspondingly, no measurable improvement in the growth of the rats was seen on frozen yeast A as compared with that of unfrozen

TABLE 1

Characterization of the baking yeasts tested for the availability of their thiamine.

IDENTITY OF THE YEASTS [1,2]	MANUFACTURER	TYPE YEAST	MANUFACTURERS' DECLARATION OF POTENCY [2,3]	WEIGHT OF YEAST WHICH FURNISHED 1 MG. THIAMINE
				gm.
A	1	Consumers' foil	Two cakes supply 100% of the adult minimum daily requirements for A, D, B_1, and 14 to 20% for B_2 plus 2 to 3 mg. niacin	25.2
A_2	1	Consumers' foil	Two cakes supply 100% of the adult minimum daily requirements for A, B_1, B_2, D plus 10 mg. niacin	12.6 [4]
B	2	Consumers' foil	12.0 mg. B_1 per pound 6.0 mg. B_2 per pound	37.8
C	2	Special [5]	12.0 mg. B_1 per pound 6.0 mg. B_2 per pound	37.8
D	2	Enriched [5]	90 to 114 mg. B_1 per pound 6.0 mg. B_2 per pound 800 mg. niacin per pound 600 mg. iron per pound	5.1 4.0
D_2	2	Enriched [5]	114 mg. B_1 per pound 6.0 mg. B_2 per pound 800 mg. niacin per pound 600 mg. iron per pound	4.0
E	2	High thiamine [5]	114 mg. B_1 per pound 6.0 mg. B_2 per pound	4.0
F	2	Regular [5]	3.3 to 3.6 mg. B_1 per pound 50 to 55 mg. niacin per pound 15 to 20 mg. iron per pound 6.0 mg. B_2 per pound	137.5

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[1] Yeast B was fed to human subjects in a previous experiment (Parsons and Collord, '42). Yeast F required so high an intake to supply 1–2 mg. of thiamine for human subjects that it was tested only with rats. The other six yeast samples were fed from identical lots to the human diet squads and the rats reported in these two papers.

[2] Yeast samples C, D, D_2 and E were all made from the same batches as were corresponding samples of yeast F but were modified by the manufacturers.

[3] Thiamine was added as the hydrochloride to yeasts B, C, D, D_2 and E by the manufacturer. It was not stated in what form thiamine was added to yeasts A and A_2. All the types of yeast contained a small amount of some form of starch and a trace of oil.

[4] The thiamine concentration in yeast A_2 was found by biological and chemical assay in this laboratory to be double that in yeast A, although it could not be so interperted from the label.

[5] For use by bakers.

fresh yeast (fig. 1, groups A 4 and A 5). This again was in agreement with the human tests. These results with freezing and those of the Pennsylvania Laboratory are in agreement in that the utilization of the thiamine was correlated with the viability of the yeasts in both cases.

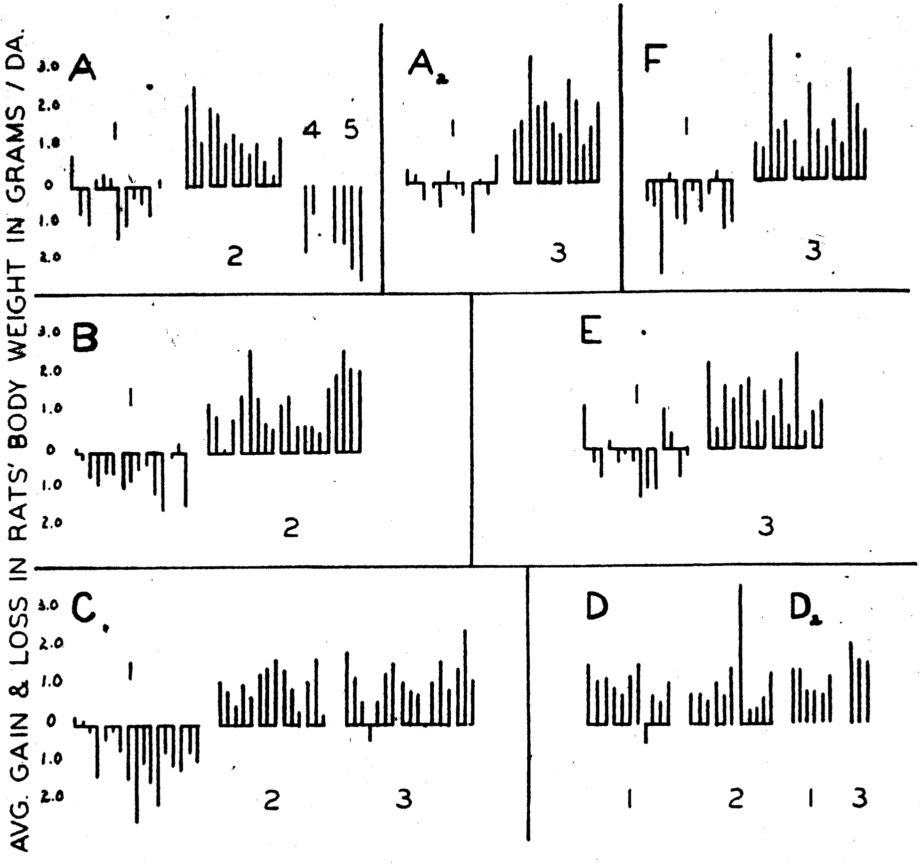

Fig. 1 Lines represent the average daily gain or loss in grams of body weight of rats on fresh and treated yeasts, actually fed in alternating periods but the records regrouped arbitrarily for comparison.

Letters refer to the brand or sample of yeast; numbers, to the treatment as follows: 1, fresh, raw yeast; 2, treated with alcohol; 3, boiled; 4, frozen in cake; 5, frozen in liquid.

Each group of lines banded together represents the repeated (although not consecutive) performances of one rat on one given yeast preparation. For example, the first line in A 1 indicates that the rat (after an initial 9-day depletion period on the basal ration) gained 0.9 gm. per day on fresh, raw yeast A and in the succeeding 10-day period on boiled yeast, gained 2.2 gm. per day (first group under A 2). Then in alternating fresh and boiled yeast periods, the rat lost 0.7 gm.; gained 2.7 gm.; lost 1.0 gm.; and gained 1.8 gm. (on the average daily). Only a part of the total assays carried out are presented but these were taken in blocks from consecutive rats' records and, hence, were unselected.

CONCLUSIONS

Biological assays of the thiamine of fresh yeasts showed that this was relatively unavailable to rats in six of the eight yeast samples tested but was rendered available for growth when the fresh yeasts were boiled or treated with alcohol.

The two samples of yeast which were nearly as effective sources of thiamine for growth when the yeast was fed fresh, as purchased, as after boiling or treatment with alcohol, were the two with the highest vitamin content of the series fed..

Short periods (24 hours) of freezing had no measurable effect in increasing the availability of thiamine from a poorly utilized fresh yeast.

These results of rat assays are in general agreement with the tests on human subjects on these same yeast samples and preparations from them.

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THE RETENTION OF THIAMINE, RIBOFLAVIN AND NIACIN IN COOKING PORK AND IN PROCESSING BACON

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The question of the retention of the B vitamins in meat during cooking and other processing is of primary importance in assessing the contribution of these vitamins to the diet by this important food group. Waisman and Elvehjem ('41) have published a compilation of the available data on the vitamin content of meat to that date. The data are largely concerned with raw meat and there is little information on the losses in cooking. Since that time several papers concerning the losses to be expected in cooking meat under specified conditions have been published. A large part of this work has been done at the University of Wisconsin (McIntire, Schweigert, Henderson and Elvehjem ('43); Schweigert, McIntire and Elvehjem ('43); and McIntire, Schweigert and Elvehjem ('43)). Additional information has been contributed by Cover, McLaren and Pearson ('44) and by Lane, Johnson and Williams ('42). Despite this work it was felt that there was a need for further data by other laboratories to provide a more complete picture. The effect of variations in the cooking procedure on the retention of the vitamins has not been fully investigated, other workers having used only optimal conditions. Exact cooking procedures are seldom used in the average household and the effect of common errors in cooking on the retention of vitamins must be understood to arrive at a figure for retention representative of less exact procedures. Cover, McLaren and Pearson ('44) gave this matter some consideration when they reported the retentions in rare and well done beef, but no results of this nature have been reported for pork.

This paper is the report of an investigation of the amount of thiamine, riboflavin and niacin in various pork cuts, the variation to be expected between carcasses and between cuts from the same carcass, the retention of these vitamins in roasting and frying under optimal conditions, and the effect of variations from these optimal conditions. There is included some information on the retention of these vitamins in the processing of bacon.

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[1] Under the direction of Alan Brown, M. D., F.R.C.P. (Lond.).

METHODS OF ASSAY

The samples were of a size that would be handled ordinarily in domestic use. The entire sample was ground finely in an electric meat grinder and thoroughly mixed by hand. A 100-gm. sample of this mixture was then weighed out and homogenized with 400 ml. of 0.1 N HCl in a Waring blendor. This blended sample was kept in the refrigerator for assay. Tests showed that the sample could be preserved in this way for at least 6 weeks with no detectable loss of thiamine, niacin or riboflavin. Samples of this meat-acid mixture were weighed out for assay of the individual vitamins.

Thiamine was assayed by the thiochrome method (Hennessy, '41; Hennessy and Cerecedo, '39). Some difficulty was encountered in preparing a suitable extract containing all the thiamine in the meat. Lane, Johnson and Williams ('42) have reported similar difficulties. At first a simple extraction with dilute acid at 100°C. followed by digestion with takadiastase at pH 4.5 was tried. When this extraction procedure was used to test the keeping properties of thiamine in the meat-acid mixture, it was found that there was a gradual increase in the apparent thiamine. This increase was more marked if the meat-acid mixture was preserved at room temperature than when it was kept in the refrigerator. This observation pointed to the presence of some complex of thiamine not extractable in the free form by this procedure but gradually decomposed on standing. Various enzymes were tried in addition to the takadiastase to effect this decomposition, and the following extraction procedure was evolved. A sample of the meat-acid mixture was weighed out in a small beaker. One hundred mg. of pepsin per gram of meat was dissolved in water to make a 5% solution and added to the meat-acid sample. This was mixed and incubated for 4 hours at 37°C. At the end of this time the sample was mixed with 50 ml. of 0.1 N HCl and the mixture steamed for 1 hour in an Arnold steamer, cooled, and the pH adjusted to about 4.5, using 2.5 M sodium acetate solution. Two hundred mg. of takadiastase and 200 mg. of maltase, dissolved together in 10 ml. of water, were added to the mixture and incubated over night at 37°C. The solution was then diluted to 100 ml. and filtered. Higher values for thiamine were obtained with this procedure and there was no evidence of liberation of further amounts from the meat-acid mixture on standing. Recovery of thiamine added to the meat-acid mixture ranged from about 90 to 100% for a series of tests. The average recovery was 94.5%. The thiamine content of the various enzyme preparations was so small that it was neglected without introducing an appreciable error.

Riboflavin was assayed by the microbiological assay method of Snell and Strong ('39). Extracts were prepared by autoclaving with 0.1 N HCl, neutralizing to pH 5.0 to 5.5, and filtering cold. There was a voluminous precipitate at this pH which effectively entrapped any fatty material. The precipitate carried down a small amount of riboflavin, approximately 10% of the total. Accordingly, the precipitate was re-extracted by autoclaving with 0.1 N HCl, neutralizing and filtering. It was washed several times with water and the combined extracts and washings adjusted to pH 6.8, diluted to an appropriate volume, and filtered again. The filtrates were crystal clear and there was no evidence of interference by fatty materials. No further riboflavin could be found in the precipitate. Loss of riboflavin in standard solutions heated under the same conditions of temperature, time and acidity was only about 2%.

A preliminary comparison of the chemical and microbiological assay procedures for niacin was made before the selection of an assay method for this constituent. The chemical assay method used was that of Dann and Handler ('41), with a few minor modifications. The hydrolysis with hydrochloric acid was modified by heating at 15 pounds steam pressure for ½ hour instead of heating on the water bath for 1 hour. The adsorption on Lloyd's reagent, elution, and clarification with lead nitrate was not changed. The development of the color was modified to use half the amounts of clarified extract and reagents, maintaining the same relative concentrations. The heating period with CNBr, was 10 minutes exactly at 75°–80°C., the temperature not being allowed to fluctuate outside this maximum range. The 5% metol reagent was made up in 5% acetic acid. The addition of the acetic acid resulted in an augmented color production. The color was read on an Evelyn photoelectric-colorimeter, using Filter 400. Recovery of added niacin ranged from 90 to 104% in ten experiments, and averaged 94.2%.

The microbiological assay was that of Snell and Wright ('41) as modified by Krehl, Strong and Elvehjem ('43). Several extraction procedures were tested. The first of these consistd simply of extraction in 0.1 N hydrochloric acid by autoclaving at 15 pounds for 1 hour. The mixture was cooled, neutralized to pH 6.8, diluted to volume and filtered. The second procedure used the same acid extraction. The mixture was then cooled and made approximately 0.1 N with respect to NaOH by addition of 10 N NaOH. It was then heated to boiling and allowed to cool to room temperature. The alkali was neutralized to pH 6.8 with HCl and the extract diluted to volume. This procedure had previously been used with cereals to hydrolyze the alkali labile niacin complex present in that type of material. The third procedure consisted of heating on a boiling water bath for ½ hour in 4% NaOH. The alkali was then neutralized to pH 6.8 with hydrochloride acid, the mixture diluted to volume and filtered. A portion of the clarified filtrate used for the color development step of the chemical assay was appropriately diluted and assayed by the microbiological procedure.

TABLE 1

Comparison of microbiological and chemical assays for niacin in meat — (µg./gm.).

ASSAY METHOD	EXTRACTION METHOD	BEEF				PORK			
		Raw		Cooked		Raw		Cooked	
		Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
Microbiological	0.1 N acid extraction	53.5	62.5	99.0	81.0	34.3	34.0	51.0	46.3
	0.1 N acid extraction then hydrolyzed in 0.1 N NaOH	52.2		101.0		35.1		53.0	
	4% NaOH extraction	52.7		101.0		34.2		52.0	
	Acid hydrolysis and clarification as for chemical assay	57.0	62.2	96.1	77.0	34.9	34.6	50.5	50.2
Chemical		62.5	65.0	101.0	82.8	40.5	39.4	56.3	54.6

Table 1 shows comparative results by these methods on some beef and pork samples, both raw and cooked. The cooked samples appearing in this table were made by heating the ground raw meat in a covered cast iron frying pan until all the liberated juices had been concentrated to the point that they could be absorbed again on the meat. The meat was then reground and thoroughly mixed. It was found that all the extraction procedures gave comparable results with the microbiological assay, well within the experimental error of the assay method. However, the chemical procedure gave results that were higher than those obtained by the microbiological method, with the exception of one sample of cooked beef. The discrepancy was more marked with the pork than the beef samples. The fact that the microbiological assays on the same clarified extracts used for the chemical assays were lower than the chemical assays and comparable with the microbiological assay results obtained on other extracts indicates that the higher values obtained with the chemical method were not due to liberation of extra niacin by the more vigorous treatment used in the chemical procedure for preparation of extracts. The agreement between the microbiological assays on the clarified extracts and the other extracts is good evidence of the specificity of the biological response, since it is probable that any foreign materials that might influence the biological response given by the relatively crude extracts prepared by the first three methods, would be eliminated by the treatment with Lloyd's reagent and precipitation with lead nitrate used for the preparation of the clarified extract. Furthermore, the agreement between microbiological assays by all four extraction procedures indicates that all the procedures probably extract the niacin completely.

The microbiological assay method was selected for use in this investigation, the 0.1 normal acid extraction procedure being used. However, eleven raw pork samples and six cooked pork samples were assayed by both the chemical and microbiological procedures. It was found that the chemical procedure was higher by an average of 45% on the fresh pork and 25% on the cooked pork.

The B vitamins in raw pork

Information was first obtained on the variations in the B vitamins that could be expected between various samples of raw pork. This was required in order that some estimate could be made of the significance of the variations in the vitamin content of the cooked meat samples and their raw controls.

The variation between cuts from a single carcass was determined by dividing a carcass into its commercial cuts. Each cut was boned and the meat was ground and mixed. The variations observed between the different cuts, with the exception of the tenderloin, were not marked. The mean variation of the thiamine was ± 9%, that of the riboflavin was ± 4%, while the mean variation of the niacin was ± 9%. However, the tenderloin contained a much higher content of all the B vitamins than any other cut. The thiamine, riboflavin and niacin content of the tenderloin was 57%, 50% and 45%, respectively, higher than the mean of the same vitamin for the rest of the carcass.

Table 2 shows the variations for picnic hams and butts between various carcasses and between opposite sides of the same carcass. The meat cuts used for analysis were commercially trimmed, i.e., the skin was removed and some of the fat trimmed. The weight of the picnic hams averaged 3100 gm., while that of the butts averaged 3700 gm. The series was too small to warrant an extensive statistical analysis; however, the following calculations were made. The average variation of the thiamine content of the individual hams from the mean thiamine value for all the hams was approximately ± 19%. Similarly the average variation for the butts was ± 36%. The same variation of the riboflavin content was ± 6% for the hams and ± 4% for the butts, while the variation of the niacin content was ± 15% for the hams and ± 20% for the butts. The variation of the right and left cuts of a carcass from the mean value for the carcass was also calculated, and the mean value of these variations for all the carcasses was determined. This value for the thiamine in picnic hams was found to be ± 7%, and was ± 2% for the butts. The value for this variation for riboflavin was ± 3% in the hams and ± 1.5% in the butts, while that for the niacin was ± 14% in the hams and ± 3% in the butts. It can thus be seen that the variations between carcasses were much more than the variations between the sides of a single carcass. Calculation of the assay results on a fat-free basis did not decrease the variations of the thiamine and niacin values and considerably increased that of riboflavin. Therefore, all later results were calculated on the basis of the whole sample and no attempt was made to correct for variations in the fat content of the samples.

TABLE 2

Variations between carcasses and between sides of the same carcass. (μg./gm. fresh meat basis).

CUT	CARCASS NO.	RIGHT SIDE				LEFT SIDE			
		Fat	Thiamine	Riboflavin	Niacin	Fat	Thiamine	Riboflavin	Niacin
		%				%			
Picnic ham	2	25.7	9.83	1.28	38.9	24.1	7.04	1.25	43.2
	3	25.3	7.30	1.25	42.0	31.0	8.11	1.19	31.6
	4	24.3	...	1.07	42.6	24.1	8.93	1.25	33.0
	5	16.5	6.53	1.21	44.5	25.5	6.4	1.22	26.8
Butt	6	27.9	5.3	1.14	29.5	28.3	5.45	1.16	33.0
	7	32.3	10.7	1.17	44.2	24.3	10.2	1.24	41.5
	8	27.3	6.9	1.25	35.6	33.3	6.2	1.25	34.6
	9	29.7	7.2	1.22	33.9	24.9	7.5	1.26	36.3
	10	30.2	5.3	1.22	29.0	29.0	5.3	1.24	27.4

The B vitamins in cooked pork

The small variations noted between butts from the two sides of a single carcass led to the selection of this cut for the sample used in the experimental determination of the losses of the B vitamins in the roasting of pork. The butts were divided in halves to limit further the variations and to obtain roasts of a size commonly used domestically (about 1800 gm.). The halves were numbered 1 and 2, the numbers referring to anatomically the same half in each case. No. 1 half-butt from one side and no. 2 half-butt from the other side of a carcass were then roasted while the remaining halves were reserved as raw controls. The six butts from three carcasses were used for the series for each type of roasting. Mean retention values for each series can thus be considered significant to within ± 2% for thiamine, ± 1.5% for riboflavin, and ± 3% for niacin, on the basis of the small series in table 2. Actually a figure of about 5% for all the vitamins would probably be better, as the series in table 2 was too small to warrant a close interpretation of the statistical analysis.

TABLE 3

The retention of B vitamins in roast pork.

		TIME OF COOKING IN MIN. PER LB.	SHRINK.	THIAMINE			RIBOFLAVIN			NIACIN		
				Raw	Cooked	Retention	Raw	Cooked	Retention	Raw	Cooked	Retention
			%	µg./gm.	µg./gm.	%	µg./gm.	µg./gm.	%	µg./gm.	µg./gm.	%
Correctly cooked	Max.	45.0	43.0	6.78	5.98	68.0	1.30	2.12	113.0	26.5	38.0	88.5
	Min.	35.0	30.0	5.35	3.58	38.0	1.12	1.46	84.5	23.5	23.4	65.0
	Avg.	40.0	38.5	6.36	4.75	46.0	1.18	1.82	96.0	25.1	31.0	78.0
Underdone	Max.	35.0	44.0	7.78	7.15	74.5	1.34	2.19	123.0	29.0	38.0	121.0
	Min.	19.0	25.6	3.09	2.13	35.5	1.15	1.91	90.0	12.6	19.3	72.0
	Avg.	26.3	36.3	5.54	5.08	57.5	1.25	2.06	105.0	20.8	30.3	94.0
Overdone	Max.	40.5	50.0	6.83	4.34	45.0	1.20	2.20	107.0	29.2	41.0	99.0
	Min.	33.0	34.0	3.35	2.10	28.2	1.13	1.73	80.0	15.5	19.3	70.0
	Avg.	36.7	43.7	5.19	3.42	36.8	1.18	1.93	92.0	20.7	29.0	75.2

The first cooking experiments were designed to determine the losses due to roasting pork. A digest of the results of these experiments is given in table 3. Three methods of cooking were investigated. The half-butts were boned and rolled before cooking in all cases. The first method, labelled "correctly cooked" in the table, consisted of roasting the pork in an open pan in an oven at 350°F. until the internal temperature at the center of the roast reached 185°F. The next cooking method, labelled "underdone," was designed to illustrate the results of attempting to hurry the cooking by using a hotter oven. For experimental purposes the meat was roasted in an open pan at 450°F. until the exterior was nicely browned. The internal temperature of the roast at this time was 150°F.–155°F. and the interior was distinctly underdone. The third method of cooking, labelled "overdone," comprised roasting in an open pan at 400°F. until the internal temperature reached 190°F. By this time the exterior of the roast was just beginning to char. Roasting was done in the oven of an ordinary domestic electric stove. Oven temperature was controlled by a thermostat. The accuracy of the temperature setting of the thermostat was checked with a standard mercury thermometer. The roasts were weighed before and after cooking and the loss in weight was calculated as the per cent shrinkage. The individual roasts were then ground and mixed for sampling. The mean values and the maximum and minimum values are reported in the table. The vitamin content is expressed as micrograms per gram of the ground sample as weighed out. The retention was found by calculating the vitamin content of the cooked samples on a raw basis by multiplying by $\frac{100 - \%\ \text{shrink}}{100}$ and comparing this figure with the vitamin content of the corresponding raw control. The necessity of determining the dry weights of all samples and calculating the results on a dry weight basis was thus eliminated.

The mean retentions of the three B vitamins that were investigated were found to be highest in the underdone roasts and lowest in the overdone roasts. There was little or no loss of riboflavin. The mean retentions varied from 92% in the overdone meat to 105% in the underdone meat. The loss of niacin was also quite small. However, about half the thiamine was lost, the mean retentions ranging from 36.8% in the overdone roasts to 57.5% in the underdone roasts.

The retention of the B vitamins in frying pork chops was investigated next. Samples for frying and for raw controls were selected by the following procedure. An entire pork loin was sliced into chops about ¾-inch thick. As the chops were cut off they were placed successively in one of three piles. The first chop was placed in pile no. 1, the second in pile no. 2, the third in pile no. 3, the fourth in pile no. 1, the fifth in pile no. 2, the sixth in pile no. 3, and so on until the chops from the entire loin had been divided among the three piles. One pile was ground and mixed to serve as the raw control for the other two piles from the same loin. These piles were fried, ground and sampled separately. There were about eight individual chops in each pile.

The ranges and mean values of the data obtained from the experiments on frying chops are given in table 4. The effect of overcooking and undercooking was again investigated. The cooking method designated as "correctly cooked" was recommended by Canada Packers, Ltd., and was found to produce a well cooked juicy product. The frying pan was heated until it just began to smoke. The chops were browned on one side and the grease was then discarded. The chops were then browned on the other side and cooking completed by heating in the open pan in the oven at 325°F. for 15 minutes. The undercooked chops were simply browned rapidly in a hot pan. The total cooking time was about 10 minutes and the interiors of the chops were underdone but not raw. The overdone chops were browned rapidly on both sides in a hot pan and then covered and cooked on the top of the stove for about 20 minutes. These chops were considerably shrunken and dried.

TABLE 4

The retention of B vitamins in fried pork chops.

		SHRINK.	THIAMINE			RIBOFLAVIN			NIACIN		
			Raw	Cooked	Retention	Raw	Cooked	Retention	Raw	Cooked	Retention
		%	μg./gm.	μg./gm.	%	μg./gm.	μg./gm.	%	μg./gm.	μg./gm.	%
Correctly cooked	Max.	46.0	8.24	8.06	58.5	1.45	2.12	81.0	21.0	38.7	103.0
	Min.	34.5	5.15	4.69	53.0	1.46	1.72	72.5	23.9	32.9	88.0
	Avg.	39.0	6.70	6.25	56.8	1.46	1.84	76.9	22.5	35.5	96.6
Underdone	Max.	32.5	8.68	9.49	87.5	1.49	2.17	103.0	23.1	32.4	123.0
	Min.	29.5	5.83	6.49	73.5	1.33	1.63	82.5	18.2	30.5	93.0
	Avg.	31.2	7.26	8.16	78.0	1.41	1.87	91.1	20.7	31.8	107.0
Overdone	Max.	48.0	6.20	5.64	52.0	1.38	2.05	78.0	24.2	46.0	111.0
	Min.	44.5	5.09	4.86	42.0	1.46	1.77	64.5	21.6	30.9	68.0
	Avg.	46.6	5.65	5.12	48.8	1.42	1.92	75.1	22.9	38.6	91.0

Here again, as in the case of roast pork, the less cooking the meat received the greater the retention of the vitamins. The retention of thiamine ranged from a mean of 78% in the underdone chops to 48.8% in the overdone chops. The extent of this variation in retention was somewhat greater than that found for roast pork. The retention of riboflavin ranged from a mean of 91.1% to a mean of 75.1%, while that of niacin ranged from 107% to 91%. It is interesting to note that the retention of thiamine and niacin is greater in fried chops than in roast pork receiving a similar degree of cooking, while the retention of riboflavin is lower.

The B vitamins in bacon

The results of an experiment designed to determine the retention of the B vitamins following the curing and smoking of bacon are reported in table 5. The experiment was undertaken in cooperation with Canada Packers, Ltd., and the curing and smoking methods used were their standard processes. The "wet cure" processing of sides used for the production of "Devon" brand bacon and the "dry" or "box" cure processing of sides and backs used for the production of "Maple Leaf" brand bacon, were investigated.

TABLE 5

The retention of the B vitamins in processed bacon.

PROCESS		THIAMINE			RIBOFLAVIN			NIACIN		
		Fresh	Processed	Retention	Fresh	Processed	Retention	Fresh	Processed	Retention
		µg./gm.	*µg./gm.*	%	*µg./gm.*	*µg./gm.*	%	*µg./gm.*	*µg./gm.*	%
Wet cure (Devon) side	Max.	6.14	4.59	84.0	1.30	1.12	102.0	34.8	25.6	90.5
	Min.	3.40	2.48	67.0	0.99	1.09	79.0	18.3	16.6	68.0
	Avg.	4.41	3.53	74.1	1.15	1.10	89.0	25.4	21.9	80.8
Dry cure (Maple leaf) side	Max.	4.66	3.99	96.0	1.34	0.91	63.5	24.3	23.8	115.0
	Min.	2.43	2.16	75.5	1.22	0.68	45.5	16.8	21.4	78.5
	Avg.	3.68	3.40	83.0	1.29	0.80	55.4	20.8	22.3	97.0
Dry cure (Maple leaf) back	Max.	11.5	8.07	109.0	1.72	1.13	62.0	39.5	47.3	109.0
	Min.	5.83	6.66	62.5	1.61	0.96	52.0	32.3	31.2	86.0
	Avg.	8.18	7.37	85.0	1.66	1.08	57.9	37.0	40.1	95.9

Retention calculated on 89% yield of bacon for the dry cure for both side and back, and 92% yield for the wet cure side. These figures are for average yields as supplied by Canada Packers, Ltd.

Four pieces of pork were selected for testing each process. Two of these pieces were selected from the shoulder end of the full sides or backs, and the remaining two were chosen from the ham ends. Each piece was about 18 inches long. About 5 to 6 inches of each piece was cut off and ground raw to serve as the control. The remaining piece was carried through the process and ground for the processed sample. The rind was removed in all cases.

The "wet cure" gave the lowest retention of thiamine, with an average of 74.1%. The "dry cure" gave 83% retention for the sides and 85% retention for the backs. The retentions of riboflavin show the greatest difference between the "wet" and "dry" cures. The retention by the "wet" cure was 89%, while the "dry" cure retained only

55.4% and 57.9% in the sides and backs, respectively. The retention of niacin was best by the "dry" cure, being nearly 100% for both the side and back bacon, whereas 80.8% was retained by the "wet" cure. Hence somewhat better retention of thiamine and niacin is obtained by the "dry" cure processing of bacon, but the "wet" cure process retains a much larger percentage of the riboflavin.

DISCUSSION

The raw samples of pork assayed throughout the study showed the following content of the B vitamins. The thiamine content of eight loin samples averaged 6.95 μg./gm., with a maximum range from 5.15 μg./gm. to 9.06 μg./gm. The riboflavin content of the same samples averaged 1.36 μg./gm. and ranged from 1.09 to 1.49 μg./gm., while the niacin content averaged 26.3 μg./gm. and ranged from 18.2 to 42.6 μg./gm. Twenty-nine samples of raw pork butts showed an average thiamine content of 6.22 μg./gm., with a range of 3.09 to 10.7 μg./gm. The riboflavin content averaged 1.21 μg./gm., with a range of 1.12 to 1.34 μg./gm., and the niacin content averaged 30.8 μg./gm., with a range of 12.6 to 44.2 μg./gm. The loins appeared to be somewhat higher than the butts in thiamine and riboflavin and slightly lower in niacin, but the ranges overlapped considerably for all three vitamins.

McIntire, Schweigert, Henderson and Elvehjem ('43) report the assays on fresh loin and fresh ham control samples. Their assays for thiamine ranged from 7.7 to 14.8 μg./gm. for hams, and 7.4 to 15.2 μg./gm. for loins. Riboflavin ranged from 2.1 to 3.4 μg./gm. for hams, and 1.7 to 3.0 for loins. Niacin ranged from 31 to 38 μg./gm. for hams, and 31 to 49 for loins. These ranges for thiamine and riboflavin are distinctly higher than those found by us, the riboflavin values, particularly, being approximately twice as high. The range reported for niacin falls in the upper limits of the range we have found. The higher content of thiamine and riboflavin found in raw pork by the Wisconsin group may be the result of differences in feeding methods used for growing and fattening hogs in the United States and Canada. Miller, Pence, Dutcher, Ziegler and McCarty ('43) report an increase of 100% in the thiamine content of pork muscle when the thiamine intake was increased from 1.318 mg. to 3.447 mg./pound of feed.

Comparison of the retention figures reported by McIntire et al. ('43) with those we have found reveal a further difference. They report an average retention of 68% of the thiamine in roast pork, while we found a retention of 46% for pork that had been correctly cooked by our methods. Even pork that was obviously undercooked retained only

57.5% of the thiamine. However, the retentions of riboflavin and of niacin agree fairly well with the retention of 90% of the riboflavin and 79% of the niacin reported by these workers.

The retention of thiamine in fried pork chops was found to be higher than that in roast pork butts. This observation parallels that made by Schweigert, McIntire and Elvehjem ('43) on roasted and fried ham. These workers also observed a higher retention of niacin by frying, although the difference they noticed was not as large as we observed. However, we observed a lowered retention of riboflavin by frying, which was not found in the work on ham. It must be realized that this work on cured ham is not strictly comparable to our work on fresh pork. The difference between our observations and those of the Wisconsin group on the retention of thiamine in roast pork point to the desirability of further study on this important subject.

CONCLUSIONS

The variation between cuts from a single carcass (with the exception of the tenderloin) and between the right and left sides of a carcass is much less than between different carcasses.

The more the pork is cooked, whether it is roasted or fried, the lower is the retention of thiamine, riboflavin, or niacin.

The greatest loss occurs in thiamine in either roasting or frying, the losses of riboflavin or niacin being comparatively small.

Frying gives a higher retention of thiamine than roasting.

The "wet cure" method of processing bacon gives a lower retention of thiamine and niacin than the "dry" or "box cure" but gives a much higher retention of riboflavin.

ACKNOWLEDGMENT

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DENTAL CARIES IN THE COTTON RAT[1]

III. EFFECT OF DIFFERENT DIETARY CARBOHYDRATES ON THE INCIDENCE AND EXTENT OF DENTAL CARIES

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Shaw et al. ('44, '44 a) have reported a high incidence and extent of carious lesions in the molars of cotton rats fed purified rations high in sucrose. In direct contrast, a low incidence and extent of caries were observed in those cotton rats fed natural rations or purified rations where the sucrose was replaced by coarse or fine dextrin. Since such a definite difference was obtained by the replacement of the sucrose by dextrin, an investigation was carried out to determine the effect of several different carbohydrates in purified rations on the incidence and extent of carious lesions. The results of these studies and the growth data obtained from these experiments and those previously reported on dental caries are presented in this paper.

EXPERIMENTAL

The cotton rats used were obtained from our stock colony. Those on the carbohydrate experiments were weaned at a weight of 15–20 gm. rather than at a weight of 20–25 gm. as was done in the previous work. This change was made since it appeared that the animals were able to adapt themselves more readily to the experimental conditions at the earlier age. As nearly as possible males and females were equally divided within each experimental group. Similarly, members of each litter were distributed equally between all groups in any experiment. After the animals had been on experiment for 14 weeks, they were sacrificed for the observation of the incidence and extent of tooth decay. The techniques of observation and evaluation of the carious lesions were the same as described in the earlier work (Shaw et al., '44, '44 a).

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The percentage composition of the sucrose basal ration 801 is as follows: sucrose 73, casein 18, salts IV 4, corn oil 5; and adequate quantities of the B vitamins (McIntire, Schweigert and Elvehjem, '44) were provided. Each rat was given one drop of halibut liver oil per week. Ration 802 was prepared by replacing 6% of the sucrose in ration 801 with casein, which brought the protein level to 24%.

The other carbohydrate diets were prepared in a similar manner by replacing the sucrose with either maltose,[2] glucose,[3] lactose,[4] dextrimaltose[5] (both with and without added NaCl), fructose,[6] fine dextrin, coarse dextrin or soluble starch.[7] The water of crystallization of glucose (cerelose) and maltose was taken into account in the preparation of these rations. The addition of 4% of 1: 20 liver extract had been shown previously to increase the growth rate (McIntire et al., '44), and not to alter the incidence or extent of dental caries (Shaw et al., '44). Therefore 4% of 1: 20 liver extract was added, at the expense of the entire ration, to all the rations used in the carbohydrate experiments.

TABLE 1

Comparison of the rate of growth of males and females over a 6-week period.

RATION	MALES		FEMALES		PER CENT DIFFERENCE A-B/B × 100
	No. of animals	Grams gain per wk. (A)	No. of animals	Grams gain per wk. (B)	
802	14	6.9	18	5.6	23
802 + 4% 1: 20 L.E.	20	9.0	11	7.2	25

In table 1 a summary of the data on the growth rate of males and females obtained from thirty-two rats fed the sucrose basal ration 802 and from thirty-one animals fed ration 802 + 4% 1: 20 liver extract is presented. It is obvious that the males consistently grew at a more rapid rate. In order to express the growth data as one figure for each group the values for the females were increased by 25%. Since only a limited number of animals were available for experiments, it was not possible to use animals of one sex in any single experiment.

[2] C.P. hydrate, Pfanstiehl Chemical Company.

[3] Cerelose, Corn Products Refining Company.

[4] U.S.P., Merck and Company.

[5] Mead Johnson and Company.

[6] d-Levulose C.P. special, Pfanstiehl Chemical Company.

[7] Reagent grade, Pfanstiehl Chemical Company.

Various fractionations of 1: 20 liver extract were made and the fractions were fed to cotton rats which received rations 801 and 802. All the liver fractions were added at the expense of the entire ration. Data on the rate of growth are included for groups of animals from earlier dental caries studies in order to compare the growth results with the severity of tooth decay. The average gains per week for both the first 6 weeks and 14 weeks on experiment are presented in table 2. The incidence and extent of carious lesions obtained when the different carbohydrate diets were fed are shown in table 3.

RESULTS

When rations 801 and 802 were fed, the average rate of growth in the first 6 weeks was approximately the same, namely 6.7 and 6.9 gm. per week, respectively. The rate of growth was more uniform when ration 802 was fed; therefore, 24% casein was used in all rations subsequent to this observation. When whole liver substance, 1: 20 liver extract or the alcohol extract of 1: 20 liver extract were added to ration 801, the growth rate was increased to 10.3, 8.1, and 7.7 gm. per week, respectively. Similarly solubilized liver, 1: 20 liver extract, or the alcohol extract of 1: 20 liver extract increased the growth rate when added to ration 802, to 8.2, 9.0, and 7.8 gm. per week, respectively.

The growth rate and ability of the cotton rat to survive were markedly affected by the particle size of the complex carbohydrates. When fine dextrin, soluble starch or fine stock rations were ingested, inferior growth was obtained. This was largely attributed to the fact that the animals scattered these fine rations which resulted in low food consumption. It can be seen in table 2 that seven of the eight animals fed the fine dextrin ration, which contained the 18% casein, died within 6 weeks. In another series when 1: 20 liver extract was added to the extent of 4% and the casein increased to 24%, all four cotton rats survived the 14-week experimental period but the growth was still poor. However, all nine animals died when the soluble starch ration, which contained 4% 1: 20 liver extract and 24% casein, was fed.

In contrast to these results coarse dextrin diets afforded excellent growth, in fact better growth than with any of the other carbohydrates (table 2). Animals which received the maltose, sucrose, glucose, fructose or dextri-maltose rations grew at approximately the same rate, 8.6, 9.0, 8.4, 8.0, and 7.8 gm. per week, respectively, for the first 6 weeks on experiment and 5.6 ,6.5, 6.0, 5.7 and 6.0 gm. per week for the entire 14-week period.

The incidence and extent of carious lesions were high when the soluble carbohydrates were ingested (table 3). These results are in direct contrast to the low incidence and extent observed when animals received coarse or fine dextrin rations. No appreciable difference was demonstrated between the coarse and fine dextrin rations. The replacement of 50% of the sucrose by fine dextrin did not reduce the severity of the tooth damage as compared to that observed when the sucrose basal

TABLE 2

Growth of cotton rats fed various diets.

(All results expressed as growth of males for reasons given in the text.)

RATION	NO. OF ANIMALS	RATE OF GROWTH PER WEEK 6 weeks	RATE OF GROWTH PER WEEK 14 weeks
		gm.	*gm.*
801[1]	13	6.7	4.9
801 + 4% 1: 20 liver extract (L.E.)	7	8.1	6.1
801 + alcohol — ether extract ≅ to 9% 1: 20 L.E.	8	6.2	4.1
801 + alcohol extract ≅ to 6% 1: 20 L.E.	3	7.7	6.5
801 + 4% whole liver substance	2	10.3	..
801 + additional vitamins ADEK	9	4.8	3.2
Sucrose replaced by coarse dextrin	9	6.4	3.1
Sucrose replaced by fine dextrin	8[2]	2.3	..
802	32	6.9	4.5
802 + 4% 1: 20 L.E.	31	9.0	6.5
802 + 4% Sol. L.E.	7	8.2	5.8
802 + alcohol extract ≅ to 6% 1: 20 L.E.	8	7.8	4.7
802 + acetone extract ≅ to 6% 1: 20 L.E.	3	6.7	..
803 (soluble starch) + 4% 1: 20 L.E.	9[3]	..	..
804 (glucose) + 4% 1: 20 L.E.	7	8.4	6.0
805 (dextri-maltose) + 4% 1: 20 L.E.	5	7.8	6.0
806 (½ of the sucrose replaced by lactose) + 4% 1: 20 L.E.	4	6.1	4.2
807 (½ of the sucrose replaced by fine dextrin) + 4% 1: 20 L.E.	4	7.8	6.1
808 (coarse dextrin) + 4% 1: 20 L.E.	5	10.1	8.2
809 (fine dextrin) + 4% 1: 20 L.E.	4	7.0	4.4
810 (maltose) + 4% 1: 20 L.E.	5	8.6	5.6
811 (fructose) + 4% 1: 20 L.E.	4	8.0	5.7
812 (¾ of the sucrose replaced by fine dextrin) + 4% 1: 20 L.E.	4	7.8	4.0
Steenbock stock ration	6	8.3	5.0
Coarse stock ration	3	6.2	
Fine stock ration	3	5.3	
Dog Food stock ration	13	9.0	4.3

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[1] Percentage composition of diet 801: sucrose 73, casein 18, salts IV 4, corn oil 5; adequate quantities of B vitamins were provided as supplements (McIntire, Schweigert and Elvehjem, '44).

[2] Seven out of 8 animals died in 6 weeks.

[3] All animals died within 3 weeks after they were placed on experiment.

ration was fed. The replacement of 75% of the sucrose by fine dextrin afforded partial protection. Apparently one-sixth of the diet as sucrose is sufficient to cause a relatively high incidence of caries.

It can readily be seen that the number of lesions observed when the control diet (802 + 4% 1:20 liver extract) was fed varied somewhat between groups; the average incidence observed in this series of experiments ranged from 23.5 to 33.0 caries per rat. These values are of the same magnitude as observed in the earlier work. The average extent of the lesions varied from 74 + to 113 +. These differences are largely due to the variation in susceptibility to tooth decay of offspring from different parent stock. Therefore, it is important that each litter be equally represented in each group of any experiment. Records have been kept of the litters represented in each experimental series. An investigation of the susceptibility of different strains of cotton rats to caries formation is now in progress and the results from this investigation will be reported later.

TABLE 8

The effect of different carbohydrates on the incidence and extent of carious lesions.

RATION	NUMBER OF ANIMALS	AVERAGE INCIDENCE OF CARIOUS LESIONS	AVERAGE EXTENT OF CARIOUS LESIONS
802 + 4% 1: 20 liver extract (L.E.)	2	32.5	112 +
808 (coarse dextrin) + 4% 1: 20 L.E.	2	5.5	10 +
809 (fine dextrin) + 4% 1: 20 L.E.	4	10.3	16 +
802 + 4% 1: 20 L.E.	3	25.3	74 +
807 (½ of the sucrose replaced by fine dextrin) + 4% 1: 20 L.E.	4	27.7	96 +
802 + 4% 1: 20 L.E.	3	33.0	113 +
812 (¾ of the sucrose replaced by fine dextrin) + 4% 1: 20 L.E.	4	23.5	50 +
802 + 4% 1: 20 L.E.	3	32.3	94 +
805 (dextri-maltose) + 4% 1: 20 L.E.	3	32.7	100 +
805a (dextri-maltose + 2% NaCl) + 4% 1: 20 L.E.	2	36.0	94 +
802 + 4% 1: 20 L.E.	2	23.5	77 +
804 (glucose) + 4% 1: 20 L.E.	7	27.6	95 +
806 (½ of sucrose replaced by lactose) + 4% 1: 20 L.E.	4	22.5	64 +
802 + 4% 1: 20 L.E.	3	26.3	77 +
810 (maltose) + 4% 1: 20 L.E.	5	27.6	81 +
811 (fructose) + 4% 1: 20 L.E.	4	29.8	92 +

DISCUSSION

The soluble carbohydrates appear to favor conditions for the formation of carious lesions while dextrin, a carbohydrate which is not readily fermented by lactic acid bacteria or other acid-producing organisms, does not favor such conditions. Unfortunately animals which received the soluble starch did not survive the experimental period, but this carbohydrate would not be expected to favor conditions for extensive tooth decay. The replacement of one-half of the sucrose by dextrin did not result in a decrease in the caries incidence. A similar result was obtained with dextri-maltose, which contains approximately 50% dextrin. Since the carbohydrate was the only dietary constituent varied in these experiments, its importance in the etiology of dental caries in the cotton rat must be emphasized.

Boyd ('42) concluded that carious lesions in children were arrested and prevented by the ingestion of diets whose quality was significantly superior to the diet of the average child. The ingestion of considerable quantities of sugar did not result in an extension of the caries or development of new carious lesions in a few cases where the effect of high quantities of sugar was specifically studied. In a later paper Boyd ('43) reported similar results from a study conducted over a 17-year period on the incidence and progression of carious lesions in children with diabetes mellitus. These experiments show that dental caries in humans can be controlled by the diet. Jay ('40) has suggested that the carbohydrate foods in the diet are largely responsible for decayed teeth.

The mechanism by which soluble carbohydrates favor conditions for a high caries incidence in the cotton rat may be much more complex than merely affecting conditions in the oral cavity. More extensive studies and many approaches to the problem will be necessary before the role of the dietary carbohydrates can be accurately evaluated.

SUMMARY

1. Data on the growth rate of cotton rats maintained on various carbohydrate diets and purified rations are presented. Additional growth responses could be demonstrated when either solubilized liver, 1:20 liver extract or whole liver substance was added to the sucrose rations.

2. Dextri-maltose, glucose, fructose, maltose and sucrose diets afforded approximately the same rate of growth. Fine dextrin, stock or soluble starch rations when fed to cotton rats produced inferior growth as compared to the sucrose control ration.

3. A very high incidence and extent of carious lesions were noted when glucose, dextri-maltose, fructose, maltose or lacto-sucrose diets were fed. However, fine dextrin and coarse dextrin diets did not favor conditions for severe tooth decay and a very low incidence and extent of the lesions were observed. There was no appreciable difference in the results obtained with coarse or fine dextrin.

4. The replacement of one-half of the sucrose with fine dextrin did not reduce the severity of tooth decay as compared to animals which received the sucrose diets. The replacement of three-quarters of the sucrose with fine dextrin reduced the incidence and extent to a small degree.

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THE RELATION BETWEEN ABSORBED NITROGEN, NITROGEN BALANCE AND BIOLOGICAL VALUE OF PROTEINS IN ADULT DOGS [1]

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TWO FIGURES

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The biological value of a protein is generally defined as the fraction of absorbed food nitrogen retained in the body of an animal, a concept proposed by Thomas and developed primarily by Mitchell ('24, '44). An objection to the use of this value has been the calculation of it which involves the assumption that excretion of nitrogen from "endogenous" sources is constant (Mitchell, '44). This concept of biological value, however, is fundamentally a function of the relationship between nitrogen balance and nitrogen intake, a relationship, which, without assumptions, can be used to evaluate dietary proteins. One of the most direct approaches to the establishment of a correlation between nitrogen balance and nitrogen intake was made by Melnick and Cowgill ('37) in their determination of protein minima for nitrogen equilibrium in dogs. They found a linear relationship in the region of nitrogen equilibrium between nitrogen balance and per cent of protein calories in the diet. The following work was done on normal adult dogs to establish more clearly over a wider range of values the relation between absorbed food nitrogen, nitrogen balance, and biological value of proteins.

METHODS

Table 1 records the protein free diet used in these studies; it is a diet similar to the one used by Melnick and Cowgill ('37). Except when otherwise noted the dogs received 80 cal./kg. of body weight daily. Protein was included in the diet by replacing equivalent amounts of calories from glucose and/or dextrin. When a natural foodstuff containing carbohydrate and fat as well as protein was added, the diet was

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adjusted so that the caloric intake was still constant and the relative proportions of constituents not altered significantly. The flounder and chicken entrails and the flounder heads used in these studies were collected fresh, ground, cooked for 1 hour in a pressure cooker at 10 pounds steam pressure, cooled and then frozen. The hearts, gizzards and livers were removed from the chicken entrails, the remainder being washed free from ingesta before grinding and cooking.

TABLE 1

Composition of protein free diet.

PROTEIN FREE DIET			VITAMIN SUPPLEMENTS	
	Per kilogram body weight *cal.*	*gm.*		*mg./kg./day*
Sucrose	14.0	3.50	Thiamine	0.025
Dextrin	12.6	3.15	Riboflavin	.025
Glucose	21.4	5.35	Nicotinic acid	.250
Lard	32.0	3.55	Calcium pantothenate	.200
Salt[1]	...	0.30	Pyridoxine	.015
Agar	...	0.40	Choline	15.0
	80.0	16.25[2]	2-Methyl-Naphthoquinone	.00001
			Calcium α-tocopherol monosuccinate	1.0
			Navitol (Squibbs)	I.U.: 4700 of Vit. A I.U.: 850 of Vit. D

[1] Wesson's modified Osborne-Mendel salt mixture.

[2] Mix 1.4 gm. of water with every gram of dog food.

The rations were fed for 8 days, the feces (with markers) and urine being collected the last 4 days. The feces and the urine samples were pooled respectively for each dog and analyzed for nitrogen by the Pregl micro Kjeldahl method.

Six adult dogs, parasite free, in good health and weighing from 6 to 10 kg. were used in these experiments.

RESULTS

The relationship between nitrogen balance and absorbed nitrogen when casein was the source of dietary nitrogen for the six dogs is illustrated in figure 1-A. Both nitrogen balances and absorbed nitrogen values are expressed as grams per day per square meter of body surface. The relationship is linear in the region of negative balance, the linearity extending over into the positive side but becoming obviously curvilinear well on the positive side of nitrogen balance.

The empirical equation for the linear portion of this relationship is,

$$NB = k\ (AN) - NE_o \qquad (1)$$

where NB is nitrogen balance, AN absorbed nitrogen, NE_o the excretion of nitrogen on the protein free diet, and k the slope of the line. Thus the slope of the line is the rate of change of nitrogen balance with respect to absorbed nitrogen, and is one measure of the biological value of the protein. This slope is constant over the linear portion becoming a variable which decreases with increasing nitrogen intake over the curvilinear portion of the curve.

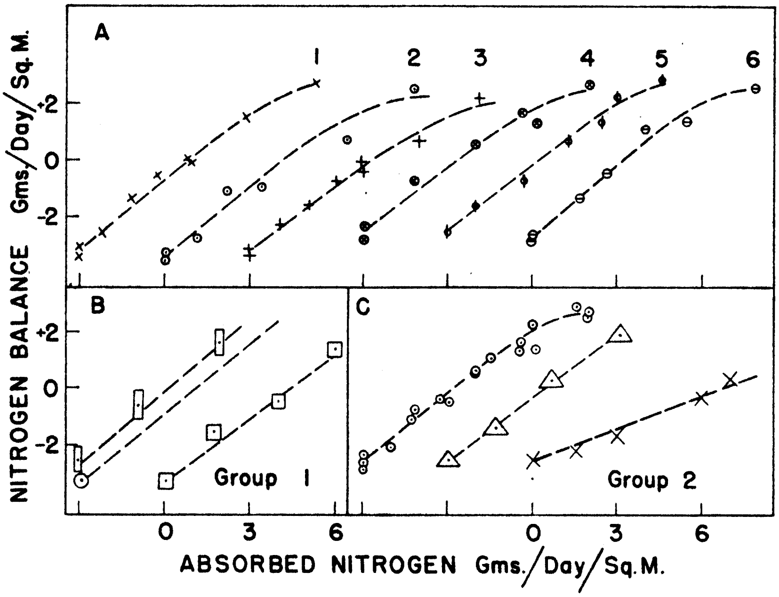

Fig. 1 A plot of absorbed nitrogen vs. nitrogen balance. All curves originate at 0 absorbed nitrogen. Figure 1-A describes data obtained on dogs 1–6 fed casein as the source of nitrogen. Figure 1–B and C describes average data obtained on dogs 1, 2, and 3, labeled group I and 4, 5, and 6, labeled group II. See text for further description.

If certain assumptions are made, the slope or k in equation (1) is the fraction of absorbed food nitrogen retained in the body of an animal, the biological value as generally defined. This definition of biological value (BV) can be illustrated by equation 2.

$$BV = \frac{AN - (UN - UN')}{AN} \qquad (2)$$

where AN is absorbed nitrogen, UN the excretion of total urine nitrogen and UN′, the excretion of urine nitrogen of "endogenous" sources.

Absorbed nitrogen is calculated according to equation 3

$$AN = NI - (FN - FN') \tag{3}$$

where NI represents nitrogen intake, FN total feces nitrogen, and FN′ feces nitrogen of "endogenous" origin.

The equivalent of AN in equation 3 can be substituted into the numerator of 2, so that,

$$(BV)\ (AN) = NI - FN - UN + FN' + UN' \tag{4}$$

Since by definition,

$$NB = NI - FN - UN \tag{5}$$

then,

$$(BV)\ (AN) = NB + UN' + FN' \tag{6}$$

Assuming that the total excretion of nitrogen on a protein free diet (NE_o) is equal to the sum of nitrogen of "endogenous" origin in urine and feces (UN′ + FN′), then,

$$NB = (BV)\ (AN) - NE_o \tag{7}$$

Equation 7 is identical to equation 1 except that k in 1 now has the definition of biological value generally used. Thus, if the assumption is made that "endogenous" nitrogen excretion is constant, then the slope of the line relating nitrogen balance to absorbed nitrogen becomes the fraction of food nitrogen retained in the body.

Values for nitrogen excretion on the protein free diet together with absorbed nitrogen at equilibrium and biological values for each of the six dogs are recorded in table 2. The biological values recorded in the last column in the table may be considered constant, since the deviations from the mean are not significant. These values are the slopes of the lines in figure 1-A. If it is assumed that "endogenous" nitrogen is constant, they represent the fraction of absorbed nitrogen retained by each animal; otherwise they are simply the rate of change of nitrogen balance with respect to absorbed nitrogen. Under absorbed nitrogen at equilibrium in the third column, are values which represent the protein minima for nitrogen equilibrium. These minima are approximately 4 gm. for the first three dogs and 3.1 gm. for the second three. The difference in minima between the two groups of dogs is correlated with the greater excretion of nitrogen on the protein free diet by dogs 1, 2, and 3, than by 4, 5, and 6, which can be interpreted to mean that the first three dogs were excreting more nitrogen from body stores requiring more absorbed protein nitrogen to maintain equilibrium. These observations agree with those of Melnick and Cowgill ('37) who noted that the lines relating nitrogen balance to per cent protein caloric intake tended to be parallel, while protein minima varied. More recent experiments have shown that increasing the caloric intake by adding carbohydrate to the diet of dogs 1, 2, and 3 decreases the excretion of nitrogen on a protein free diet bringing the data into line with the other three dogs.

The first two curves in figure 1-B illustrate this effect of increasing the caloric intake of dogs 1, 2, and 3, labeled group I. The second dotted line originating at the circle represents the average curve for the data on the first three dogs fed casein in a diet of 80 calories per kilogram of body weight (figure 1-A). The first line through the rectangles is an average curve for the same three dogs when the caloric intake was increased to 100 calories per kilogram of body weight. The slopes, and therefore the biological values of casein at the two caloric intakes, are identical. The only change is a shift in the excretion of nitrogen, being lower on the higher caloric intake, thus demonstrating the protein sparing action of carbohydrate. The fact that the excretion of nitrogen can shift without altering the biological value supports the concepts developed by Mitchell ('24, '44).

TABLE 2

Nitrogen excretion on a protein free diet (NE_0), absorbed nitrogen at nitrogen equilibrium and the biological value (BV) taken from each curve in figure 1-A. Casein was the source of dietary nitrogen.

DOG NO.	PROTEIN FREE NITROGEN EXCRETION NE_0	ABSORBED NITROGEN AT EQUILIBRIUM		BIOLOGICAL VALUE BV
	gm./day/sq.M.	*gm./day/sq.M.*		
1	3.2	3.8		0.84
2	3.4	4.0		0.85
3	3.3	4.2		0.78
4	2.5	3.1		0.81
5	2.5	3.1		0.81
6	2.7	3.2		0.84
			Average	0.82

The first curve in figure 1-C averages the same data for casein obtained on dogs 4, 5, and 6, labeled group II, as plotted in figure 1-A. The slope of this line is 0.82 the same as the slopes of the lines for casein in group I. The line through the triangles describes average data obtained using chicken entrails as the source of protein nitrogen in dogs 4, 5, and 6. The slope is 0.77, which is slightly less than that for casein. The line through the crosses represents data for a commercially prepared protein derived from soybean, with a slope of 0.39, much lower than the others. The squares in the figure 1-B summarize data obtained on dogs 1, 2, and 3, using flounder entrails as the source of protein in the diet, the slope of the line being the same as that for chicken entrails. Table 3 summarizes some of these and other averaged data on dogs in groups I and II.

TABLE 3

Digestibilities, absorbed nitrogen at nitrogen equilibria, and biological values (BV) of casein, protein A and proteins in chicken entrails, flounder entrails, and flounder heads. Data are averages from determinations on two groups of three dogs each, I and II.[1]

DOG GROUP	PROTEIN SOURCE	TRUE DIGESTIBILITY	ABSORBED NITROGEN AT EQUILIBRIUM	BV
		%	*gm./day/sq.M.*	
I	Casein	96	4.0	0.82
I[1]	Casein	95	3.2	0.81
II	Casein	95	3.1	0.82
II	Chicken entrails	94	3.3	0.77
I	Flounder entrails	96	4.3	0.77
I	Flounder heads	89	6.4	0.52
II	Protein A[2]	82	6.6	0.39

[1] All dogs received 80 calories per kilogram of body weight except those in the experiments in this group when 100 calories per kilogram of body weight were fed.

[2] Derived from soybean.

Equation 1 is useful, therefore, in determining directly the biological value and the protein minima for nitrogen equilibrium. But the relationship between urine nitrogen excreted and absorbed nitrogen is also of fundamental interest. This relationship can be derived from equation 1 or 7 in the following way. The symbols are the same as those used previously.

$$NB = AN + FN - FN' - UN - FN \qquad (8)$$

Substitute 8 in 7, expanding NE_o in 7 to $UN_o + FN_o$, so that,

$$AN - FN' - UN = (BV)\ (AN) - UN_o - FN_o \qquad (9)$$

Assume $FN_o = FN'$ then,

$$UN = (1 - BV)\ AN + UN_o \qquad (10)$$

Where equation 1 or 7 described a linear relationship between NB and AN, equation 10 also describes a linear relationship between UN and AN as illustrated in figure 2. The symbols used in figure 2 are the same as those in figure 1-B, C. The circles in the lower left hand part of figure 2 describe the same data for casein as illustrated in figure 1-C for dogs in group II. Similarly the triangles and the crosses describe respectively data for chicken entrails and the derived soybean proteins.

The squares illustrate the data obtained on dogs in group I when they were fed fish entrails as the source of protein nitrogen. The slopes of the lines in this figure are equal to (1 — BV). When BV is equal to unity then urine nitrogen excretion would be independent of absorbed nitrogen, the excretion remaining the same as on a protein free diet.

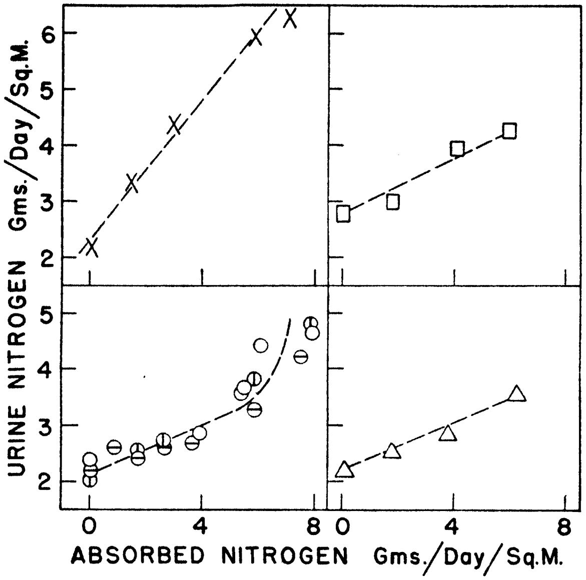

Fig. 2 A plot of absorbed nitrogen vs. urine nitrogen using data from the same experiments illustrated in figure 1–B and C.

Under these conditions UN_0 in equation 10 could be determined without feeding a protein free diet. If biological value is the fraction of nitrogen retained in the body of an animal, then BV can never be greater than unity, since the retention of more than 100% of the absorbed nitrogen is an impossibility. On the other hand, if "endogenous" nitrogen metabolism is not constant, BV will not represent the fraction of absorbed nitrogen retained and could be greater than unity. Under these conditions urine nitrogen could decrease as absorbed nitrogen increases, an example of food nitrogen sparing body nitrogen.

SUMMARY

1. The relationship between nitrogen balance and absorbed nitrogen in normal adult dogs is linear in the region of negative nitrogen balance, the linearity often extending over onto the positive side, but becoming obviously curvilinear well on the positive side of nitrogen balance.

2. The equation describing the linear portion is,

$$NB = k\ (AN) - NE_0$$

where NB is nitrogen balance, AN absorbed nitrogen, NE_0 the excretion of nitrogen on a protein free diet and k the slope of the line. If the excretion of nitrogen of endogenous origin is constant, k is the fraction of nitrogen retained in the body of an animal. This fraction is the customary definition for the biological value of a protein.

3. When the caloric intake was reduced below optimum, excretion of nitrogen on a protein free diet (NE_0) and protein minima for nitrogen equilibrium increased, but the biological value (k) was not altered. Thus reduced caloric intake increased the utilization of body nitrogen but did not alter the utilization of dietary nitrogen.

4. The value for k was determined for a variety of protein sources in the adult dog as follows: casein = 0.82, chicken entrails = 0.77, flounder entrails = 0.77, flounder heads = 0.52, and a protein derived from soybean = 0.39.

5. The relationship between urine nitrogen (UN) and absorbed nitrogen (AN) was shown to be,

$$UN = (1 - BV)\ AN + UN_0$$

where BV is biological value, and UN_0 the excretion of urine nitrogen on a protein free diet.

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NUTRITIONAL VALUE OF YEAST PROTEIN TO THE RAT AND THE CHICK

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This laboratory (Stubbs, Noble, and Lewis, '44; Lewis, Stubbs, and Noble, '44) has been interested for some time in the utilization of cannery wastes for the production of yeast for use in feeds.

One phase of this project has been the nutritional evaluation of the protein of the yeast produced. Feeding experiments with rats recently reported from this laboratory (Klose and Fevold, '44) have shown that yeast, when fed as the sole source of protein at levels as high as 13% crude protein, supplies an inadequate amount of methionine for optimum growth in the young rat. This work has been confirmed and extended in a series of rat and chick growth tests, the results of which are given here.

The literature concerned with the amino acid content and nutritional value of the proteins of yeasts has been adequately reviewed by Carter and Phillips ('44). However, some of the recent work that bears most directly on our studies will be mentioned here. Temperton and Dudley ('41) fed groups of thirty laying pullets a practical cereal ration in which the protein concentrate was supplied by fish meal (10%), torula yeast (12%), or brewer's yeast (12%). No differences between the three groups were observed as regards egg production, general health, or body weight. Feeding experiments with poultry, summarized by Axelsson ('41), indicated that at least half of the usual quantity of animal protein can be replaced by yeast protein.

Growth tests with rats were carried out by Hock ('42) to determine to what extent animal protein can be replaced by yeast protein. Determinations of the average gain in weight per gram of protein consumed were made for rats on diets containing 9.3% crude protein, of which 1.5% was derived from cereals and 7.8% from various combinations of fish meal and yeast. Essentially the same results were obtained for brewers' yeast and for torula yeast grown on wood-sugar. The growth rate was not affected appreciably by substitution of either brewers' yeast or torula yeast protein for half of the fish-meal protein; 75% replacement reduced the gain 15 to 25%, while complete replacement of fish meal by yeast resulted in a 50% decrease in weight gain per unit of protein consumed.

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A recent report by Hock and Fink ('43) describes rat-growth tests in which yeast protein was beneficially supplemented with cystine. The percentage of total crude protein in the diets was in most cases 10%, of which 1.6% was furnished by cereals and the remainder (8.4%) by either brewers' yeast or wood-sugar yeast. Addition of 0.2% of cystine to the diets containing yeast resulted in an approximate doubling of the growth rate. The cystine-supplemented yeast diet had a growth-promoting effect nearly equal to that of a diet in which 90% of the yeast protein was replaced by milk protein. During the experimental period of 90 days, all of the yeast-fed groups experienced liver damage and heavy mortality. However, the mortality was considerably lower in the groups that received cystine.

The amounts of the essential amino acids in whole yeast, reported by Block and Bolling ('43)[2] as percentages of the crude protein (N × 6.25), were as follows: arginine 4.3, histidine 2.8, lysine 6.4, tryptophane 1.4, phenylalanine 4.1, threonine 5.0, leucine 13.2, isoleucine 3.4, valine 4.4, cystine 1.3, and methionine 2.0. Values for the percentage of cystine in the crude protein (N × 6.25), of dried whole yeast ranging from 0.6 to 2.2 have been reported (Prunty, '33; Csonka, '35; Fink and Just, '42). Reporting on the basis of crude protein (N × 6.25) neglects the presence of nonprotein nitrogen (purines, pyrimidines, etc.), which has been calculated from Van Slyke nitrogen fractionations, and other means, to represent approximately 15 to 20% of the total nitrogen present in yeast. Most nutritional data have been expressed on the basis of crude protein content, since the available information is insufficient to characterize completely a large majority of protein concentrates in terms of their component proteins and nonprotein nitrogen constituents. It appears advisable in the present work to adhere to this conventional basis of comparing the nutritional value of protein concentrates, at the same time keeping in mind the discrepancies involved and the corrections that may be applied when more data become available.

EXPERIMENTAL WORK

All sources of protein were fed on the basis of their crude protein content (total Kjeldahl nitrogen × 6.25). The yeast sample designated as "brewers' yeast" was a commercial type grown on a hop-free grain medium. It had a crude protein content of 44%. Torula yeast, sample A,[3] consisted of Torulopsis utilis grown on a prune-juice medium. The yeast cells were separated by pressing, dried in ribbon form in a tunnel dehydrator, and then ground in a hammer mill. Torula yeast sample B represented a similar product prepared from blackstrap molasses. Samples A and B contained 57.5 and 53.2% crude protein, respectively. Sample B was found to contain 0.1% cystine.[4] Of the reference proteins used, casein had 84%, sardine meal 70%, and soybean meal 44% crude protein. A 50% methanol extract of brewers' yeast was prepared by leaching the yeast with three successive volumes (2.5 ml. of solvent per gram of yeast) of the extractant and concentrating in vacuo to a thick syrup. This protein-free concentrate was added to those diets that contained little or no yeast in order to minimize the possibility of an uncompensated growth effect from some nonprotein factor in the yeast.

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[2] Also see Melnick, '43.

Growth tests with rats

The general procedure used in all of the rat-growth experiments to be described was as follows: Albino rats of the Sprague-Dawley strain were maintained on a stock diet of Purina Dog Chow pellets, supplemented with cod liver oil and lettuce, until the experimental diets were fed. All experimental groups contained equal numbers of males and females. Feed and water were furnished ad libitum, and a record was kept of the daily feed consumption for each cage. The rats were weighed individually at frequent intervals and examined at the end of the experimental periods for gross manifestations of toxic effects.

Preliminary feeding experiments with young rats had shown that a dietary level of 28% torula yeast A gave only one-third of the growth rate produced by a 16% level of commercial casein. Therefore experimental diets were designed to test the adequacy of yeast protein when fed as the sole source of protein and when fed in combination with casein. The ten groups of experiment A, each consisting of sixteen, 38-day-old rats, were placed on the diets described in table 1. Groups IV a and VII a were limited to a test period of 10 days; all other groups of experiment A were maintained on their respective diets for a 5-week period. The average initial weight of the rats of all groups was about

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[3] The samples of torula yeast were made available through the generous cooperation of Dr. E. C. Saudek and Mr. W. M. Noble of Consumers Yeast Company and Drs. I. C. Feustel, H. Humfeld and J. C. Lewis of this laboratory.

[4] We are indebted to D. K. Mecham of this laboratory for the analysis. A modification of Vassel's method was used (Mecham, '43).

73 gm. No deaths occurred during the experimental periods. A denudation, appearing first around the ears and later extending over the shoulders and back, was noted in the rats of groups IV and VII after 3 weeks on the experimental diets. Otherwise all rats were healthy and showed no macroscopic evidence of toxic effects upon being sacrificed and examined at the conclusion of the test. The resultant growth data given in table 1 demonstrate that, at a total crude protein level of 13.4%, neither of the two yeasts tested could support growth comparable to that obtained with an equivalent amount of crude casein protein. One-fourth of the casein, however, could be replaced by yeast without appreciable effect on growth rate. The good growth obtained when yeast was supplemented with methionine (groups IV a and VII a) demonstrates the nature of the deficiency in yeast protein.

TABLE 1

Comparative growth promoting effect of protein from yeast and from casein.

	EXPERIMENT A									
	Diet no.									
	I	II	III	IV	IVa	V	VI	VII	VIIa	VIII
Basal mixture,[1] %	65	65	65	65	65	65	65	65	65	65
Starch-sucrose (50-50), %	19	23	27	5	5	12	15.5	10	10	14.5
Yeast extract (protein-free), %	≅ 5	≅ 5	≅ 5	..	..	..	..	..	..	..
Commercial casein, %	16	12	8	..	..	8	12	..	..	8
Brewers' yeast, %	..	..	..	30	30	15	7.5	..	..	..
Torula yeast B, %	..	..	..	..	..	..	..	25	25	12.5
dl methionine, %	..	..	..	..	0.5	..	..	..	0.5	..
Crude protein from casein, %	13.4	10.0	6.7	..	..	6.7	10.0	..	..	6.7
Brewers' yeast, %	..	..	..	13.2	13.2	6.6	3.3	..	..	..
Torula yeast B, %	..	..	..	..	..	..	..	13.3	13.3	6.7
Average gain in weight, gm./rat/day[2]	2.5	1.6	0.4	0.6	3.3	1.9	2.3	0.6	3.2	2.0
Feed consumption, gm./rat/day	10.9	10.5	8.1	9.1	9.3	10.9	10.4	8.2	9.8	11.0
Gm. gain/gm. crude proteins consumed	1.71	1.54	0.68	0.47	2.70	1.30	1.69	0.58	2.5	1.33

[1] The composition of the basal mixture used in the diets was as follows: As per cent in whole diet, sucrose 25, corn starch 25, cottonseed (Wesson) oil 10, salt mixture (McCollum's no. 185) 4, U.S.P. cod-liver oil 1, choline chloride 0.05; and as mg. per 100 gm. in whole diet, thiamine chloride 0.2, riboflavin 0.5, pyridoxine 0.2, calcium pantothenate 2.5 and nicotinic acid 1.0.

[2] Comparable groups of rats on stock colony diet gained 3.5 gm./rat/day.

A second rat growth test (table 2, experiment B) was set up in order to determine to what extent cystine could correct the methionine deficiency in yeast protein, and to make further comparisons between casein and the yeast protein. Each group contained six males and six females, with an average initial age of 40 days and average initial weight of 83 gm. Experimental diets were fed over a period of 37 days.

TABLE 2

Growth responses obtained with methionine and cystine supplements to a yeast diet, compared with responses with reference casein diets.

	RAT EXPERIMENT B									
	Diet no.									
	I	II	III	IV	V	VI	VII	VIII	IX	X
Basal mixture,[1] %	60	60	60	60	60	60	60	60	60	60
Starch-sucrose (50-50), %	15	15	15	15	15	..	27	24	24	20
Yeast extract (protein free), %	..	..	..	..	..	..	≅ 5	≅ 5	≅ 5	≅ 5
Commercial casein, %	..	..	..	..	..	..	13	16	16	20
Torula yeast B, %	25	25	25	25	25	40	..	..	..	..
l-cystine, %	..	0.5	..	..	0.5	..	..	..	0.2	..
dl-methionine, %	..	..	0.2	0.5	0.2	..	..	..	..	..
% crude protein, %	13.3	13.6	13.4	13.6	13.7	21.3	10.9	13.4	13.5	16.7
Average gain, gm./rat/day	0.8	1.0	2.0	2.4	2.7	1.7	2.0	2.7	3.1	3.1
Feed consumption, gm./rat/day	8.6	7.8	9.2	9.5	10.2	9.3	12.0	11.7	11.6	11.6
Gm. gain/gm. crude proteins consumed	0.73	0.91	1.65	1.82	1.90	0.84	1.54	1.70	1.96	1.58

[1] The composition of the basal mixture used in the diets of experiment B was as follows: As per cent in whole diet, sucrose 22, corn starch 22, cottonseed (Wesson) oil 10, salt mixture (McCollum's no. 185) 4, U.S.P. cod liver oil 2, vegetable oil concentrate containing 40% tocopherols 0.05, choline chloride 0.05; and as mg. per 100 gm. in whole diet, thiamine chloride 0.2, riboflavin 0.5, pyridoxine 0.2, calcium pantothenate 2.5, and nicotinic acid 1.0.

Yeast protein at levels of 13.3 or even 21.3% crude protein again was found inadequate for optimum growth. Supplementation with 0.5% methionine at the 13.3% crude protein level resulted in essentially the same rate of growth as was obtained with a 13.4% crude protein level of casein. Cystine had a definite supplementing effect on yeast protein, which was most striking in the group fed diet V.

A comparison of the growth rates in group VIII, IX and X demonstrates the long recognized fact that casein, when used in diets at levels below 18% crude protein, supplies a suboptimal amount of cystine.

Very little of the denudation noted in experiment A occurred in the groups of experiment B. As in earlier tests, no deaths occurred during the experimental period, in contrast to the results of Hock and Fink ('43), who reported considerable mortality during the first 5 weeks of a 90-day test period. At the conclusion of experiment B, the rats were sacrificed and examined macroscopically for fatty livers or other abnormal conditions. No liver damage or other organic lesions were apparent.

A feeding experiment with rats on a 37.5% torula yeast diet over a period covering the growth of two generations was still in progress when this paper was written. No deaths had occurred in male rats after 75 days on this diet, or in females after 90 days. Sacrifice and examination of the males after 75 days showed no abnormalities in the liver or kidneys.

Chick growth tests

Possible uses of yeast include its substitution for animal protein in poultry rations. Since the amino acid requirements of the chick are known to differ in some respects from those of the rat, it was considered desirable to investigate the effect of adding yeast to chick diets in amounts that would furnish significant contributions of proteins.

In the chick growth tests to be described, 1-day-old single-comb White Leghorn chicks were housed in thermostatically controlled, electrically heated, all-metal battery brooders and fed an adequate practical chick ration until the feeding experiments were started. Groups of chicks, selected for the same average weight and weight distribution, were fed the experimental diets ad libitum during a 12-hour day controlled arbitrarily by lighting. All diets were adjusted to contain approximately 1.6% calcium and 1.0% phosphorus. Records were kept of the individual chick weights and of the feed consumption by groups.

Chick test I. Preliminary growth studies with chicks demonstrated that 80% of the protein of the fish meal in a practical chick diet could be replaced by yeast protein without seriously affecting the growth rate or efficiency of food utilization. It was thought desirable to compare the growth-promoting value of protein from yeasts with those of fish-meal and soybean-meal protein, especially when each occurred in a cereal diet as the sole protein concentrate. Four hundred 1-day-old chicks of both sexes were maintained on an adequate practical chick ration (diet 1 in table 3) for 10 days. Then nine groups of 28 chicks each, selected from those ranging from 70 to 90 gm. in weight, were fed the diets described in table 3 for 20 days.

The resultant growth data in table 3 indicate that when used as the sole source of noncereal protein, yeast is markedly inferior to sardine meal and significantly inferior to soybean meal. This is in contrast to the satisfactory results obtained by replacing 80%, rather than 100%, of the protein of sardine meal by yeast protein. Although the differences are not highly significant, it may be pointed out that the combination of soybean meal and sardine meal appeared superior to the soybean meal alone, as has been reported by Hammond and Titus ('44). However, the combination of soybean meal and torula yeast gave a lower growth rate than the soybean meal alone. This is understandable in view of the relative methionine shortages in the two protein concentrates.

TABLE 3

A comparison of the growth produced by yeast, sardine meal, and soybean meal in chick diets.

INGREDIENT	CHICK TEST I Diet no. 1	2	3	4	5	6	7	8	9
Basal mixture,[1] %	71.5	71.5	71.5	71.5	71.5	71.5	71.5	71.5	71.5
Ground corn, %	11	10	10	..	6	4	..	5	2
Corn starch, %	..	5	10	..	..	..	..	..	..
Steamed bone meal, %	1.3	2.3	3.2	2.6	1.7	1.4	3.1	2.2	2.3
Calcium carbonate, %	1.0	0.8	0.7	1.7	2.5	2.7	1.2	1.1	2.0
Yeast extract, %	≅ 10	≅ 10	≅ 10	..	..	..	≅ 10	≅ 10	..
Sardine meal, %	15	10	5	..	..	..	..	7.5	..
Brewers' yeast, %	..	..	..	24	..	..	..	..	..
Torula yeast A, %	..	..	..	..	18	..	..	..	..
Torula yeast B, %	..	..	..	..	..	20	..	..	10
Soybean meal, %	..	..	..	..	..	..	24	12	12
Crude protein, %	19.8	16.2	13.1	19.1	19.8	19.5	19.0	19.6	19.5
Gain in weight, gm./chick/day									
Average	11.8	10.3	8.2	8.3	9.1	8.9	10.9	12.0	9.9
Stand. error	0.4	0.3	0.4	0.3	0.4	0.4	0.3	0.5	0.3
Feed consumption, gm./chick/day	25	26	23	32	30	26	28	28	29
Feed utilization, gm. gain/gm. feed	0.46	0.40	0.36	0.26	0.30	0.33	0.39	0.44	0.35

[1] The basal mixture provided, as per cent of the complete diets: ground whole corn 30, ground whole wheat 10, ground oat groats 10, wheat bran 15, alfalfa meal 5, sodium chloride containing 2% manganese sulfate 0.5, and U.S.P. cod liver oil 1.

Chick test II. A second set of feeding experiments was set up to study the methionine deficiency in yeast protein when fed as the sole source of protein in simplified chick diets.

One-day-old cockerels were fed an adequate practical chick ration for 10 days. At this time the chicks were selected for uniformity, wing-banded, and divided into groups of ten each. The average weight gains and feed consumption corresponding to the various supplements are given in table 4. It is apparent that torula yeast fed at a 40% level (21.3% crude protein) was not supplying an adequate amount of methionine for optimum growth. This condition could be corrected to varying degrees by supplements of 0.5% methionine, 1% cystine, or 5% dried egg white. It may be estimated from analyses reported in the literature that the dried egg white contributed approximately 0.2% methionine and 0.1% cystine to the diet.

TABLE 4

The effect of amino acid and protein supplements on the growth rate of chicks fed a 40% yeast diet.

CHICK TEST 11

Diet no.	Supplement to 40% yeast diet [1]	Days on diet	Average gain gm./chick/day Aver.	±	Stand. error	Feed consumption gm./chick/day	Feed utilization gm. gain/gm. feed
1	None	1–16	4.4	±	0.6	27	0.16
1a	None	1–6	4.8	±	0.6	20	0.23
2	1% glycine	7–16	4.1	±	0.7	24	0.17
3	1% cystine	1–16	7.1	±	0.6	22	0.32
4	0.5% methionine	1–16	9.8	±	0.7	24	0.40
5	5.0% dried egg white	1–14	8.5	±	0.6	24	0.35

[1] This diet contained, as per cent: torula yeast B 40, glucose 22, corn starch 22, gum arabic 8, calcium carbonate 3, tricalcium phosphate 1, sodium chloride plus trace elements 1, potassium chloride 0.5, cottonseed (Wesson) oil 1, U.S.P. cod liver oil 1, vegetable oil concentrate containing 40% tocopherols 0.05, and 2 methyl 1, 4 naphthoquinone 0.0002.

The general health and appearance of the chicks in the three tests described in this report were good. Partly because of the severe culling at the start of the tests, no deaths occurred during any of the experimental periods. Chicks on the simplified diets were rather poorly feathered and grew at a much slower rate than those on practical rations. This is a common observation with simplified diets and probably reflects the physical nature of the diet and relatively obscure nutritional deficiencies.

DISCUSSION

On the basis of Block's value of 2.0% for the methionine content of the crude protein of yeast, and 0.2% for the cystine content, we may estimate that the 25% level of torula yeast was furnishing 0.27% of methionine and 0.025% of cystine to the diet. Womack and Rose ('41) have set the methionine requirement in the rat at 0.6%, or 0.5% in the presence of an adequate amount of cystine. This provides a basis for a partial explanation of the results obtained by supplementing yeast with varying amounts of methionine and cystine. For example, in rat experiment B, the addition of 0.5% cystine to the 25% torula yeast diet (containing 0.27% methionine) gave only a very small increase in growth rate compared with the addition of 0.2% methionine. However, when we tested the relative supplementing effect of cystine and methionine at a higher basal level of methionine content, which approached the minimum methionine requirement (compare diets III, IV, and V in table 2, experiment B), we found that cystine served equally as well as methionine.

The high mortality in rats fed high levels of yeast as reported by Hock and Fink ('43) is in marked contrast with the 100% survival in our tests. Differences in the source of yeast, strain of rat, or basal diet could conceivably cause this discrepancy.

The experimental results of the chick growth tests agreed in general with the results of the rat studies. In simplified diets, levels as high as 40% torula yeast appeared to be deficient in methionine for optimum growth of the chick. A 40% yeast level should supply 0.42% methionine and 0.04% cystine to the diet. Almquist, Mecchi, Kratzer, and Grau ('42) have measured the chick's requirements and found them to be 0.55% methionine and 0.4% cystine, or the equivalent of these two amounts in methionine. The results of chick test II agreed with this formulation of the sulfur-containing amino acid requirements of the chick, since a 1% cystine supplement (incapable of satisfying the remaining 0.13% methionine requirement) gave a smaller increase in growth rate than did a 0.5% methionine supplement.

In practical cereal chick rations yeast protein effectively replaced as much as 80% of the animal protein concentrate. However, when animal protein was replaced completely by yeast protein, a definite decrease in growth rate occurred. In the field of protein concentrates, yeast may be compared most closely with soybean meal. Compared with good animal proteins, both of these proteins are seriously inadequate in respect to only one essential amino acid, methionine.

SUMMARY

1. Brewers' yeast and torula yeast grown on molasses have been fed as the principal or only source of protein in the diet of rats during the period of rapid growth. The same yeasts and also a torula yeast

grown on prune juice were fed to chicks. At the levels fed, and in comparison with equivalent amounts of relatively complete proteins, each of the three yeast preparations contained an inadequate amount of methionine for optimum rate of growth.

2. This inadequacy could be corrected by the addition of methionine or methionine-rich protein to the diet.

3. Cystine corrected the deficiency to a limited extent.

4. Yeasts at relatively high levels and over limited periods did not appear to be toxic.

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THE EFFECT OF FEEDING SUCCINYLSULFATHIAZOLE TO RATS RECEIVING PURIFIED DIETS HIGH IN CARBOHYDRATE, PROTEIN, FAT, OR PROTEIN AND FAT

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TWO FIGURES

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The incorporation of succinylsulfathiazole (SST) into otherwise adequate highly purified diets renders such diets incapable of supporting normal growth in the rat (Welch, '42). Accompanying the failure in growth are such evidences of a deficiency syndrome as alopecia, porphyrin-stained whiskers, spectacled eye, ophthalmitis, achromotrichia, leucopenia and agranulocytopenia (Daft et al., '42; Spicer et al., '42; Light et al., '42; Martin, '42 a, b; Nielsen and Elvehjem, '42; Welch and Wright, '43; Wright and Welch, '43). Normal growth and alleviation of the signs of deficiency may be brought about by the administration of "folic acid" and biotin. The deficiency state is characterized by low hepatic stores of "folic acid," biotin and pantothenic acid which return to adequate levels when "folic acid" and biotin are given (Wright and Welch, '43, '44).

Studies of the requirement of the rat for certain dietary factors when receiving SST in highly purified diets have been largely limited to the use of rations containing approximately 18% casein with the remainder of the diet composed largely of sucrose. The importance of the composition of the diet in relation to vitamin requirements has been demonstrated. In the case of thiamine, for example, it has been shown that more thiamine is required for normal growth with a diet high in carbohydrate than is required with diets high in protein or fat (Evans, Lepkovsky and Murphy, '34; Arnold and Elvehjem, '39; Wainio, '42). Larger amounts of riboflavin are required for normal growth of rats receiving diets high in fat (Mannering et al., '41).

The following experiments were designed, therefore, to study the relationship of the dietary composition to the deficiencies brought about by feeding SST to rats. It was thought possible that variations in the diet might alter the intestinal flora or otherwise modify the action of SST in such a way that additional deficiencies heretofore not noted might develop. Another possibility was that alteration in the diet might indicate in what way the factors which are affected by the feeding of poorly absorbed sulfonamides function in the normal metabolism of the rat. Observations on the growth and incidence of signs of deficiency have been supplemented by studies of the number and distribution of the intestinal flora and quantitative studies of the fecal elimination and liver storage of factors known to be involved in a deficiency induced by SST.

PROCEDURE

Albino rats weighing between 35 and 45 gm. were divided into four groups of twelve each and caged individually over wire mesh screening. Diets high respectively in carbohydrate, protein, fat, or in both protein and fat were fed ad libitum (table 1). After 2 weeks, when it was felt that the rats had become accustomed to the change in diet, 5% SST replaced an equivalent amount of sucrose in each of the diets. Weights were recorded weekly and, as signs of deficiency developed, at more frequent intervals.

TABLE 1

Composition of the diets employed.

VARIED INGREDIENTS OF BASAL MIXTURE	HIGH CARBOHYDRATE I	HIGH PROTEIN II	HIGH FAT III	HIGH PROTEIN AND FAT IV
	gm.	*gm.*	*gm.*	*gm.*
Vitamin-free casein	180	300	180	300
Sucrose	660	540	460	340
Hydrogenated cottonseed oil[1]	100	100	300	300

Each diet also contained the following (in gm.): corn oil 20, salts[2] 40, vitamins A, D, and E[3] 1, choline 1; and the following (in mg.): vitamin K[4] 10, thiamine hydrochloride 8, riboflavin 16, nicotinic acid 40, pyridoxine-hydrochloride 8 and calcium pantothenate 44.

[1] 'Primex'.

[2] Hubbell, Mendel and Wakeman ('37).

[3] Compounded as follows: Fish liver concentrate containing 450,000 U.S.P. units of vitamin A and 90,000 U.S.P. units of vitamin D per gram, 7 gm.; α-tocopherol, 2 gm.; corn oil, 41 gm.

[4] Used in the form of 2-methyl-1, 4-napthoquinone.

The bacterial flora of the feces was followed by dilution plate counts made twice a week on the feces of three rats chosen at random in each group. The total number of organisms (aerobic and anaerobic), spores (aerobic and anaerobic), streptococci, lactobacilli, and coliforms were determined, using the differential technique of Strawinski, Verwey and Munder ('45).

Determinations of "folic acid," biotin and pantothenic acid were made on 24-hour samples of feces (pooled sample of three rats in each group) at weekly intervals. One-half of each sample was digested for 24 hours at 37°C. with 2% of its weight of takadiastase in pH 4.0 acetate buffer (Cheldelin et al., '42) prior to microbiological assay for "folic acid"[1] (Landy and Dicken, '42) and pantothenic acid (Skeggs and Wright, '44). Biotin determinations were made according to the procedure of Wright and Skeggs ('44) on the remainder of the 24-hour samples after they had been autoclaved for 1 hour with 6 N H_2SO_4. In all the microbiological assays of feces p-aminobenzoic acid was included in the assay medium (10 mg. per 100 ml.) as a sulfonamide inhibitor.

TABLE 2

The fecal elimination of "folic acid," biotin and pantothenic acid in rats receiving various diets.

TREATMENT	"FOLIC ACID"				BIOTIN				PANTOTHENIC ACID			
	I	II	III	IV	I	II	III	IV	I	II	III	IV
	µg./rat/day				*µg./rat/day*				*µg./rat/day*			
No sulfonamide [1]	2.1	1.9	1.6	2.0	0.41	0.32	0.28	0.25	20.1	16.9	21.1	19.2
5% SST [2]	0.31	0.54	0.38	0.64	0.043	0.054	0.041	0.053	10.6	20.5	15.8	20.7

I = high carbohydrate diet; II = high protein diet; III = high fat diet; IV = high protein and fat diet.

[1] Results given are the average of two determinations made on the 8th and 11th days of feeding the purified diets.

[2] Results given are the average of seven determinations made at weekly intervals after the inclusion of SST in the diets.

Representative rats from each group were sacrificed at intervals throughout the feeding period and at the end of the experiment to obtain data on the liver storage of "folic acid," biotin and pantothenic acid. One-half of each liver was prepared for microbiological assay for "folic acid" and pantothenic acid by digestion in water with takadiastase (Wright, Skeggs and Welch, '45). The remaining portion of each liver was autoclaved with 6 N H_2SO_4 prior to microbiological assay for biotin. "Folic acid," biotin and pantothenic acid were determined microbiologically in the liver samples by the same procedures used in the fecal assays.

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[1] We are indebted to the Lederle Laboratories for a supply of crystalline Lactobacillus casei factor ("folic acid") used as a microbiological standard.

RESULTS

The growth data on the animals of the various groups are summarized in figure 1. During the pre-sulfonamide period of feeding the rats consuming the high fat diets grew at a more rapid rate, presumably as a result of an increased caloric intake per gram of diet consumed. When SST was included in the diet the animals consuming the high protein diets continued to gain weight and only ceased growing after consuming the 5% sulfonamide diet for 4 weeks. The animals were normal in appearance and free from evidences of deficiency until

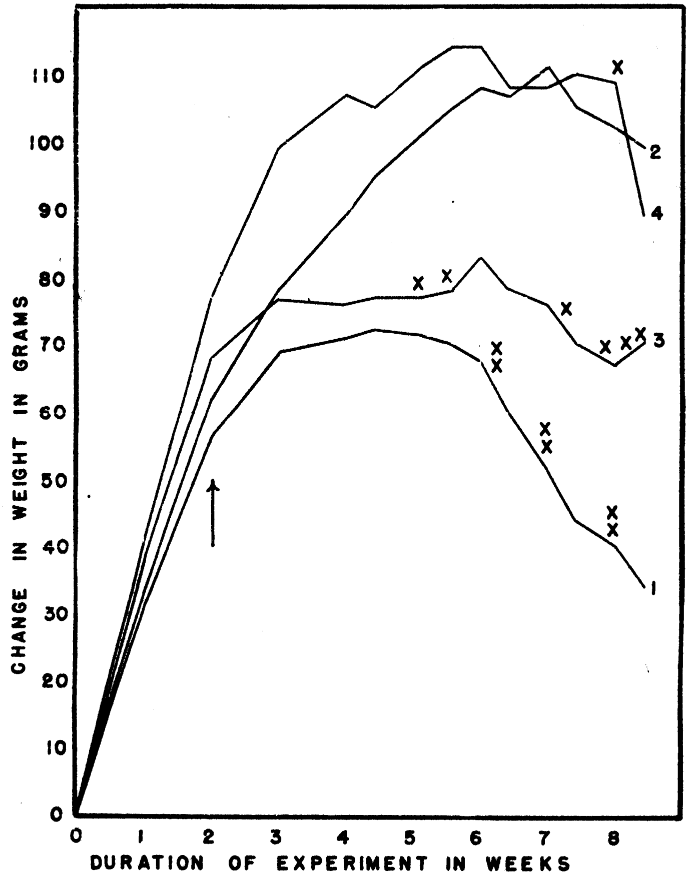

Fig. 1 Growth curves of rats receiving various diets: (1) High in carbohydrate, (2) high in protein, (3) high in fat, (4) high in protein and fat. The arrow indicates the inclusion of SST in the diets. An X indicates the death of one animal.

they began to decline in weight. In contrast, when 5% SST was included in the high carbohydrate or high fat diets growth soon ceased and all animals became untidy in appearance. Alopecia, porphyrin-caked whiskers or ophthalmitis was seen in most of the animals of these groups. Deaths were frequent in the high carbohydrate or high fat groups whereas only one animal died in the groups ingesting the high protein diets.

The bacterial counts on the feces from animals of the various groups are shown in figure 2. SST was equally effective in each of the four groups in reducing the coliform count from an initial value of approximately 40 million organisms to approximately 200 thousand organisms

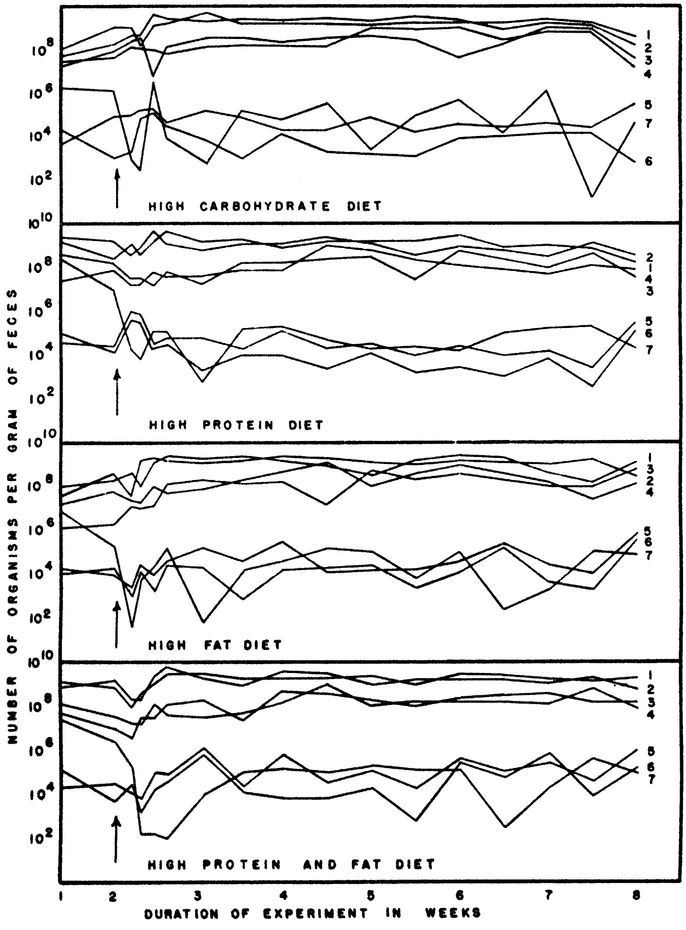

Fig. 2 Fecal elimination of bacteria as influenced by diet: (1) Total anaerobes, (2) total aerobes, (3) streptococci, (4) lactobacilli, (5) anaerobic spores, (6) aerobic spores, (7) coliforms. The arrow indicates the inclusion of SST in the diets.

per gram. The total number of organisms per gram of feces was unaffected by the inclusion of 5% SST in the various diets.

Prior to the inclusion of SST the fecal elimination of "folic acid," biotin and pantothenic acid was not affected by the composition of the diet. When SST was fed the fecal elimination of "folic acid," biotin and pantothenic acid was markedly reduced in all groups but the reduction was generally less when the diets were high in protein.

The results of occasional determinations of the liver storage of "folic acid," biotin and pantothenic acid are given in table 3. The inclusion of 5% SST in the highly purified diets was accompanied by a decrease in the hepatic storage of these factors. The stores of "folic acid" in the liver tended to be higher in those animals consuming the high protein diets. There appeared to be no difference in the storage of biotin with respect to variations in the composition of the diet. The hepatic storage of pantothenic acid was considerably reduced below that usually found in stock animals in only those groups receiving the 18% casein diets. The animals receiving the high protein diets (30% casein) appeared to maintain a normal storage of pantothenic acid until they ultimately ceased growing. The levels of pantothenic acid eventually reached in all four groups were indicative of incipient pantothenic acid deficiency (Wright and Welch, '44).

TABLE 3

The liver storage of "folic acid," biotin, and pantothenic acid in individual rats receiving various diets.

TREATMENT	DURATION OF FEEDING	"FOLIC ACID"				BIOTIN				PANTOTHENIC ACID			
		I	II	III	IV	I	II	III	IV	I	II	III	IV
	days	*µg./gm.*				*µg./gm.*				*µg./gm.*			
No sulfonamide	15	4.5	10.5	3.8	3.9	...	...	...	...	..	...	..	..
5% SST	32	0.31	0.30	0.29	0.23	0.27	0.33	0.31	0.17	57	107	58	69
5% SST	43	0.21	0.32	0.18	0.31	0.22	0.21	0.20	0.17	54	100	63	88
5% SST	61[1]	0.39	0.46	0.25	0.31	0.29	0.22	0.26	0.25	50	56	46	52

I = high carbohydrate diet; II = high protein diet; III = high fat diet; IV = high protein and fat diet.

[1] The results given are the average of data obtained on three rats.

DISCUSSION

The results obtained have shown that the composition of the diet is an important factor in the production of nutritional deficiencies by the use of SST. Diets high in protein in some manner delayed the onset of the deficiency syndrome which is induced by the inclusion of SST in the usual highly purified diets. The superiority of the diets high in protein could not be correlated with changes in the number or types of organisms present in the intestine. This would indicate that if the mechanism by which poorly absorbed sulfonamides act has a bacteriological basis, it must depend on an alteration in the metabolism rather than on changes in the actual number of the intestinal organisms present. The observations of Miller ('44) are consistent with these findings.

It seems difficult, in view of the small and sometimes inconsistent differences observed in the fecal elimination of "folic acid," biotin and pantothenic acid within the various dietary groups, to ascribe the superiority of the high protein diets solely to an increased intestinal synthesis of these factors. The hepatic storage of "folic acid" likewise, was not appreciably greater in the animals consuming high protein diets and seems insufficient to explain the growth results obtained. In the case of pantothenic acid, however, the hepatic storage in rats receiving high protein diets remained at normal levels until the rats had begun to decline in weight. Possibly the greater storage of pantothenic acid was a factor in explaining the superiority of the high protein diets.

There is much evidence that pantothenic acid is involved in carbohydrate metabolism (Williams, '43) and the requirement for this factor might reasonably be expected to be lower on a high protein than on a high carbohydrate or high fat diet. The apparent superiority of the high protein diets may actually be due to the fact that in increasing the protein content of the diet the carbohydrate has been correspondingly reduced.

These experiments have been limited to a consideration of the deficiencies of "folic acid," biotin and pantothenic acid. It is quite possible that the level of SST employed in these studies is capable of inducing additional deficiencies which are directly influenced by the level of dietary carbohydrate, protein or fat.

SUMMARY

1. A study has been made of the growth, the bacterial flora of the feces, and the fecal elimination and liver storage of "folic acid," biotin and pantothenic acid in rats fed succinylsulfathiazole (SST) containing diets high in either carbohydrate, protein, fat, or both protein and fat.

2. Weight gains continued for a longer period of time in rats fed SST in high protein diets than in animals receiving the drug in high carbohydrate or high fat diets. Some of the signs of nutritional deficiencies which early became apparent in the latter groups did not appear in animals fed the high protein diets.

3. The bacterial flora of the feces was not demonstrably altered in number or kind of organisms by changes in the composition of the diet.

4. The superior condition of the rats ingesting high protein diets showed only slight correlation with the fecal elimination of "folic acid," biotin and pantothenic acid.

5. The inclusion of SST in all the diets was accompanied by a marked reduction in the hepatic stores of "folic acid" and biotin. The stores of "folic acid" were, however, somewhat higher in the rats receiving the high protein diets.

6. The hepatic stores of pantothenic acid remained high in the animals receiving the high protein diets until they eventually began to lose weight. Of the determinations made, the hepatic stores of pantothenic acid showed the best correlation with the condition of the animals.

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